Regulation of macrophage phagocytosis of apoptotic cells by CD44

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PhD Thesis
The University of Edinburgh
1999

ABSTRACT

Development of effective therapeutic strategies for inflammatory diseases requires understanding of the cellular and molecular mechanisms that determine whether inflammation resolves or progresses to scarring and tissue destruction. Apoptosis of neutrophil granulocytes is an important determinant of the resolution of inflammation, providing a mechanism for down-regulation of function and triggering "silent" clearance by phagocytes of potentially destructive inflammatory cells. However, if the rate of cell death by apoptosis is such that macrophage clearance capacity is exceeded, apoptotic cells may progress to secondary necrosis, resulting in release of harmful cellular contents and damage to the surrounding tissue. Changes in the composition of the extracellular matrix in response to tissue injury may influence macrophage phagocytic capacity. In particular, adhesion of macrophages to fibronectin significantly augmented their capacity for phagocytosis of apoptotic neutrophils, an effect that may involve β1 integrins and other cellular receptors. The aim of this thesis was to investigate the role of CD44, a surface molecule that is expressed by many cell types including human monocytes and alveolar macrophages, in the phagocytosis of apoptotic neutrophils in vitro. Expression of CD44 by human monocyte-derived macrophages was characterised by indirect immunofluorescence and flow cytometry, and by immunoprecipitation and Western blotting. A well characterised phagocytic assay was used to assess the role of macrophage CD44 in the phagocytosis of apoptotic neutrophils in vitro. Data presented in this thesis demonstrate that pre-incubation of human monocyte-derived macrophages with CD44 monoclonal antibody 5A4 resulted in an average 2.3-fold increase in the proportion of macrophages that phagocytosed one or more apoptotic neutrophils across a range of basal phagocytosis rates, whereas control antibody had no effect. In contrast, incubation of macrophages with CD44 antibody had no demonstrable effect on phagocytosis of immunoglobulin-opsonized erythrocytes or zymosan, suggesting that CD44 ligation had specifically influenced the phagocyte machinery involved in the binding and/or engulfment of apoptotic cells. These data provide the first evidence that macrophage phagocytosis of apoptotic neutrophils can be rapidly (within minutes) augmented following ligation of specific macrophage surface receptors. CD44 ligation by antibody 5A4 had no effect on macrophage release of interleukin-8, interleukin-10, or tumour necrosis factor-α, consistent with clearance of apoptotic cells by a "non-inflammatory" mechanism. Use of bivalent $F(ab')_2$ and monovalent Fab' fragments demonstrated a requirement for cross-linking of CD44. A panel of ligands and antibodies that bind to relevant macrophage surface receptors was used to investigate whether CD44 ligation recruited previously defined mechanisms to augment phagocytosis of apoptotic neutrophils. However, none of the agents tested had any demonstrable inhibitory effect upon CD44-augmented phagocytosis of apoptotic neutrophils when used alone, suggesting that ligation of macrophage CD44 either recruits multiple apoptotic cell recognition pathways simultaneously or brings into play novel phagocytic receptors. The rapidity of effect of ligation of macrophage CD44 by antibodies suggested a direct effect on intracellular signalling pathways. In particular, examination of cytoskeletal proteins involved in phagosome formation revealed increased tyrosine phosphorylation of paxillin in response to binding of CD44 antibody. One significant finding from these studies was that CD44 ligation had no effect upon phagocytosis of apoptotic peripheral blood lymphocytes, indicating that apoptosis is associated with expression of cell-specific markers that signal recognition and phagocytosis by

macrophages. Preliminary characterisation of some of the changes on the surface of the apoptotic neutrophil that may be responsible for phagocyte recognition was therefore undertaken. Antibody and lectin binding studies suggested that relatively subtle desialation of surface carbohydrates accompanies neutrophil apoptosis. One unique monoclonal antibody, called Bob93, was identified that bound specifically to apoptotic neutrophils. Bob93 was found to bind in a sialic acid-dependent and species-specific manner to the bovine sialoglycoprotein fetuin, and further studies using labelled fetuin indicated that fetuin may bind to the surface of both human macrophages and apoptotic neutrophils. These studies provide the foundation for further analysis of the mechanisms of membrane alterations in apoptosis and the control of apoptotic cell recognition by phagocytes.

ACKNOWLEDGEMENTS

I am indebted to Chris Haslett, under whose percipient guidance this work was performed; to my colleagues in the Rayne Laboratory for their invaluable advice, especially Adriano Rossi, James Pryde, Mark Lawson, Marie-Helene Ruchaud-Sparagano, and Trevor Walker; and most of all to Ian Dransfield, for expert and comprehensive tutelage imparted with his unique blend of enthusiasm and pragmatism (and for his handy DIY tips).

This work was supported by a University of Edinburgh Faculty of Medicine fellowship.

DECLARATION

This thesis has been composed by myself and represents my own work. All of the experiments described herein were performed by myself with the exception of specimen processing for electron microscopy which was done by Steve Mitchell, and the Bob93/CD16 dual labelling data (chapter 5) which was from Ian Dransfield.

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ABBREVIATIONS

ABC	Adenosine triphosphate binding cassette
AGE	advanced glycosylation end products
ASGPR	asialoglycoprotein receptor
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CMF-PBS	calcium/magnesium-free PBS
CVF	cobra venom factor
DMB	dimethoxybenzidine
DMEM	Dulbecco's modified Eagle's medium
ECM	extracellular matrix
EIgG	immunoglobulin-opsonized erythrocytes
ELISA	enzyme-linked immunosorbent assay
ERM	ezrin/radixin/moiesin
FITC	fluoroscein isothiocyanate
FCS	foetal calf serum
GPI	glycosylphosphatidylinositol
HRP	horseradish peroxidase
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IL	interleukin
LPS	lipopolysaccharide
M-CSF	macrophage colony stimulating factor
MW	molecular weight
NRIG	non-immune rabbit immunoglobulin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PI-PLC	phophatidylinositol specific phospholipase C
PMN	polymorphonuclear neutrophil
PS	phosphatidylserine
SDS	sodium dodecyl sulphate
TGF	transforming growth factor
TNF	tumour necrosis factor

CHAPTER 1
INTRODUCTION

Injury and Inflammation

Cellular injury may result from a variety of insults, such as physical trauma, chemicals, irradiation, invading microorganisms, and immunological reactions. Injury to vascularised living tissue provokes a stereotyped series of cellular and biochemical events that comprise the acute inflammatory response, which serves to destroy, dilute, or isolate the injurious agent, and initiates mechanisms to repair the damaged tissue (Cotran et al., 1989). The cardinal clinical features of acute inflammation were recognised in the first century AD by Cornelius Celsus. Celsus, a Roman, described the characteristic rubor (redness), tumor (swelling), calor (heat), and dolor (pain) that typically followed tissue injury. It is now known that this constellation of clinical features results from vasodilatation, oedema formation, and emigration of inflammatory cells from the blood into the injured tissue - events that were first observed and described by Julius Cohnheim (1839-84) using an in vivo animal mesentery preparation. Subsequently, Elie Metchnikoff developed his theory that inflammation results in recruitment of phagocytes with the capacity to engulf microbes, and Paul Erhlich demonstrated that serum factors (antibodies) were important in the host's response to infection. In recognition of their work, Erhlich and Metchnikoff shared the Nobel Prize in 1908. Later this century, Thomas Lewis showed that chemical substances released locally following tissue injury mediated the vascular changes of inflammation (Cotran et al., 1989). This work was the prelude to immense interest in the chemical mediators that regulate cellular behaviour during the inflammatory response, and the roles of an ever increasing number of important players have now been defined (Luster, 1998).

Neutrophil granulocytes: the archetypal acute inflammatory cells

Acute inflammation is characterised by accumulation of neutrophil granulocytes. Neutrophils are leukocytes (white blood cells) that are produced in the bone marrow and circulate in the blood, from where they are recruited to a site of tissue injury following adhesion to activated vascular endothelium and transmigration into the extravascular space (Cotran et al., 1989). A series of molecular and cellular events are initiated by release of chemical mediators from the damaged tissue that lead to expression and/or activation of specialised adhesion molecules on vascular endothelial cells that bind to counter-receptors on the surface of the circulating leukocytes (Hogg and Landis, 1993). Initial tethering of the leukocytes to the endothelium is followed by rolling adhesion and then firm adhesion, each mediated by different combinations of receptor-ligand interactions. Transmigration between the endothelial cells and through the vascular basement membrane into the tissue space is directed by a gradient of chemotactic chemicals such as complement component C5a and interleukin (IL)-8 (Luster, 1998).

Once in the tissue, neutrophils are able to deploy a range of functions to destroy and remove pathogenic microbes (table I) (Savill and Haslett, 1994).

Neutrophil function	Examples
Release of granule enzymes and other proteins	elastase, collagenases, neuraminidase, heparanase, myeloperoxidase, cationic proteins
Generation of reactive oxidant mediators	H_2O_2, superoxide, singlet oxygen, hypochlorous acids
Generation of inflammatory mediators	PAF, LTB_4, cytokines
Phagocytosis	complement- and Fc receptor-mediated uptake of opsonized micro-organisms

TABLE I
Neutrophil functions at the site of tissue injury

However, inflammation is a two-edged sword, and although neutrophils are a vital component of the body's response to infectious agents, release of their formidable array of toxic substances may inflict damage on surrounding tissue and propagate the inflammatory response (Weiss, 1989). As a result, an inappropriately directed or uncontrolled inflammatory response may initiate a vicious cycle of neutrophil-mediated tissue damage and further recruitment of inflammatory cells, culminating in deposition of fibrous tissue (scarring) and impairment of organ function. Neutrophil-driven inflammation and tissue injury is thought to be a key pathological process in many important diseases such as rheumatoid arthritis, fibrosing alveolitis, the adult respiratory distress syndrome, and inflammatory bowel disease (Weiss, 1989; Jones et al., 1998). Perhaps because of the prevalence such diseases in clinical practice, it has become customary to envisage the acute inflammatory response as inevitably being superseded by destruction of tissue, chronic inflammation, and scarring. However, there are historical examples of how extensive and intense acute inflammation may resolve completely. For example, during spontaneous resolution of *Streptococcus pneumoniae* pneumonia, billions of extravasated neutrophils are completely cleared from the lung interstitium and air spaces, resulting in preservation of the delicate lung architecture and restoration of normal lung function (Savill and Haslett, 1994). Ultimately, understanding the cellular and molecular mechanisms that determine whether acute inflammation resolves or progresses to chronic inflammation and scarring may lead to the development of effective therapeutic strategies for inflammatory diseases. In recent years evidence has accumulated that the fate of neutrophil granulocytes is an important determinant of the resolution of inflammation.

Apoptosis (programmed cell death) of neutrophil granulocytes

Theoretically, release of neutrophil-derived histotoxic products is favoured by rapid recruitment, prolonged lifespan, excessive activation, or reduced clearance of neutrophils at a site of inflammation. While neutrophil recruitment and activation play undoubtedly important roles, there has been much interest in how neutrophil death may modulate the inflammatory response. Once recruited in large numbers to a site of tissue damage, neutrophils do not migrate to the lymph nodes or the bloodstream, but die locally (Savill and Haslett, 1994). Until relatively recently it was believed that neutrophils die by undergoing necrosis, the classical mode of cell death that follows irreversible cell injury (Hurley, 1983). Necrosis is associated with disruption of the plasma membrane and release of intracellular contents, which

clearly in the case of the neutrophil would promote tissue damage and cause further inflammation. However, it is now clear that neutrophils undergo the alternative, controlled mode of cell death, apoptosis.

The cell biology of apoptosis

Apoptosis is the process of programmed cell death in vertebrates, during which the cell activates intrinsic suicide mechanisms that rapidly (within hours) lead to the characteristic macroscopic features of cell shrinkage, chromatin condensation, membrane budding, and eventually formation of one or more apoptotic bodies (Kerr et al., 1972). Apoptosis and the subsequent phagocytic clearance of senescent cells are believed to play a vital role in many fundamental biological processes, including normal tissue turnover (Han et al., 1993), remodelling of embryological tissues (Hopkinson-Woolley et al., 1994), and development of the immune system (Cohen, 1991).

In the last few years substantial research activity has identified many of the molecular events involved in the apoptosis pathway (reviewed in Aravind et al., 1999, and Kinloch et al., 1999). Cell death proteins in mammals, primitive animals, and plants share a number of conserved domains. Triggering of the apoptosis pathway occurs following interaction of a cell surface receptor, such as the TNF receptor or Fas, with their respective extracellular ligands. Ligation of these receptors triggers intracellular signalling pathways, such as the classical death domain cascade that interacts with the caspases, but which may also involve a protein kinase cascade or activation of NF-κB. A further level of regulation has been revealed following the discovery of "decoy" receptors that have missing or truncated intracellular domains. Cell fate may also be determined by "survival signals" such as integrin-mediated adhesion to extracellular matrix, the absence of which may result in initiation of apoptosis.

Proteins in the apoptosis pathway bear a variety of small adapter domains (e.g., the death domain (DD), death-effector domain (DED), and the caspase activation and recruitment domain (CARD)) that interact primarily through homophilic interactions and mediate transmission of signals from the surface receptors to downstream effectors such as the caspases. Many adapter proteins are composed of two different interaction domains, for example DD-DED in FADD, that serve to link components of the apoptosis pathway together.

The inevitable consequence of initiation of the apoptosis pathway is disassembly of the intracellular machinery which is mediated by principally by the caspases, which have been regarded as the final common executioners of apoptosis. A role for cysteine proteases in apoptosis was discovered when the C. *elegans* gene product ced-3 was found to be homologous with the human interleukin 1β-converting enzyme (now known as caspase-1). Subsequently, a whole family of mammalian cysteine proteases, termed the caspases, was identified. Caspases cleave specific aspartate-containing sites in a variety of target proteins including BCL-2, BAX, IκB, and the caspases themselves, and are typically are linked to the apoptosis machinery by an adapter domain. Caspase activation may occur by two distinct mechanisms. First, adapters such as FADD or RAIDD recruit pro-caspases such as mammalian caspase-2 or caspase-8 that are bound directly to activated receptors such as the TNF receptor or Fas. The second pathway is mediated by the BCL-2 proto-oncogene family of proteins, which may be either pro-apoptotic (e.g., BAX) or anti-apoptotic (e.g., BCL-2), along with the apoptosis ATPase human APAF-1 (equivalent to C. *elegans* CED-4). Several of these proteins are associated with the outer mitochondrial membrane, and are

intimately linked with release of cytochrome c from the mitochondria into the cytosol. Apoptosis ATPases lead to activation of caspases in a complex fashion that may be dependent on cytochrome c.

In addition, a number of a serine/threonine protein kinases have been proposed to play a role in the execution of apoptosis. Calmodulin-dependent death-associated protein (DAP) kinase contains a death domain and may regulate the balance between tumour proliferation and regression (by apoptosis). RIP kinase interacts with RAIDD, downstream of TRADD, and activates a distinct apoptotic pathway. Conversely, activation of protein kinase B downstream from phosphatidylinositol-3-kinase confers protection from apoptosis in a number of cell types. The balance between life and death of the cell is also influenced by the complex mitogen-activated protein kinase signalling pathways, activation of which may be mediated by a variety of stimuli, including ceramide which is generated following cleavage of the membrane phospholipid sphingomyelin by sphingomyelinases. Furthermore, apoptosis is linked to the cell cycle machinery by the retinoblastoma RB protein and DNA-binding proteins such as p53, and the transcription factor NK-κB is linked to TNF-mediated apoptosis through adapters TRADD and TRAF and a serine-threonine kinase pathway that phosphorylates and inactivates the inhibitor IκB.

Identification of apoptotic cells in experimental systems

A variety of experimental methods have been used to identify apoptotic cells (Kroemer et al., 1997). Activation of an endonuclease during apoptosis leads to internucleosomal digestion of DNA into monomers or oligomers or nucleosomal DNA of 200 base pairs and mutiples thereof. A regular "ladder-type" pattern of oligonucleosomal DNA fragmentation on agarose gel electrophoresis is commonly considered to define apoptosis. However, this technique is clearly unsuitable for quantification of apoptosis.

DNA fragmentation also leads to an apparent reduction in nuclear DNA content, so that staining of cells with a DNA-intercalating dye such as propidium iodide allows detection of a "hypodiploid" cell population. Two dyes may be used in order to simultaneously assess the necrotic cell population on the basis of its loss of membrane integrity. Alternatively, the presence of DNA strand breaks can be assessed by enzymatic methods, since DNA breaks create acceptor sites for enzymes such as terminal deoxyribonucleotidyltransferase (TdT). Addition of TdT together with fluorescein-12-2'deoxyuridine-5'-triphosphate is used to reveal DNA fragmentation in the "TUNEL" technique.

Normal cell membranes exhibit marked phospholipid asymmetry, with phosphatidylcholine and sphingomyelin predominantly on the external layer and most of the phosphatidylethanolamine and phosphatidylserine on the inner layer. Apoptosis is associated with the loss of phospholipid asymmetry and the exposure of phosphatidylserine on the outer surface of the plasma membrane. Annexin V is a protein that binds specifically to phophatidylserine, and its fluorecent derivatives can be used to identify apoptotic cells using a simple and rapid flow cytometric method. An additional method which may be used to identify apoptotic neutrophils is based on the observation that neutrophil apoptosis is associated with a marked down-regulation of surface expression of the low affinity Fc receptor FcγRIII (CD16) (Dransfield et al., 1994). A simple flow cytometric method using a labelled CD16 monoclonal antibody reliably discriminates apoptotic from non-apoptotic neutrophils in a mixed cell population.

Clearance of apoptotic cells by macrophages

It has been established that as a consequence of poorly-characterised changes in the plasma membrane, apoptotic cells are swiftly recognised and ingested by neighbouring phagocytes (Savill et al., 1993), which partly explains why isolated apoptotic cells are rarely visualised in tissue sections by light microscopy. In fact, phagocytosis of senescent cells was first described in the late nineteenth century by the Russian biologist Elie Metchnikoff who, using a simple light microscope, observed that "microphages" (neutrophil granulocytes) were "englobed" by macrophages in injured tadpole fins. Although it is believed that in mammals "non-professional" phagocytes (e.g., hepatocytes (Dini et al., 1992), endothelial cells (Dini et al., 1995), glomerular mesangial cells (Savill et al., 1992), and fibroblasts (Hall et al., 1994)) may remove apoptotic cells in certain circumstances, the principal phagocytes are tissue macrophages.

Macrophages are tissue mononuclear phagocytes, highly specialised for endocytosis and intracellular digestion, that are derived from blood monocytes. In the tissues, macrophages acquire a phenotype that is significantly influenced by their environment, but they all retain specialised characteristics - motility; pinocytosis of soluble molecules; phagocytosis of large particles; intracellular digestion by hydrolytic enzymes; and the potential for activation (characterised by ruffled membrane, more cytoplasm, increased enzymes and lysosomes, more endocytosis, more killing ability) by lymphokines, bacterial products, or phagocytosis. The traditional role of macrophages as scavenger cells has been known for over a century, and in the inflammatory response they are abundant after the early stage, when they are responsible for engulfing foreign particles, debris, and proteins. In addition, they process and present foreign antigens to antigen-specific T lymphocytes, display cytocidal activity against tumour cells, secrete a huge array of biologically active substances, and co-ordinate the inflammatory response by generating a variety of cytokines (Auger and Ross, 1992).

Neutrophil apoptosis promotes resolution of inflammation

There is now evidence that neutrophil apoptosis is important in the resolution of inflammation. In the tissues neutrophils are stimulated to release granule enzymes, oxidants, and inflammatory mediators by external stimuli such as bacterial products, cytokines, and adhesion to matrix components and neighbouring cells via specialised adhesion receptors on the cell surface (Hogg and Landis, 1993). Neutrophil apoptosis results in loss of expression of adhesion molecules (Dransfield et al., 1995) and greatly reduced responsiveness to external stimuli (Whyte et al., 1993), so that these cells become functionally isolated from their environment. In contrast with necrosis, apoptosis is associated with preservation of plasma membrane integrity, so that release of harmful neutrophil contents is limited, and the inert neutrophil is packaged for disposal by local phagocytes. Furthermore, in contrast with ingestion of particles opsonized with antibody, phagocytosis of apoptotic granulocytes by human macrophages in vitro does not stimulate release of macrophage-derived pro-inflammatory mediators (Meagher et al., 1992; Stern et al., 1996; Fadok et al., 1998). In this way, by down-regulating neutrophil functions and triggering their "silent" clearance by phagocytes, apoptosis provides a mechanism for the safe disposal of potentially destructive inflammatory cells (figure 1). Evidence for a role for neutrophil apoptosis in the resolution of inflammation is further supported by the observation that apoptotic granulocytes are present within

macrophage phagocytic vacuoles in inflammed joints (Savill et al., 1989) and asthmatic airways (Wooley et al., 1996).

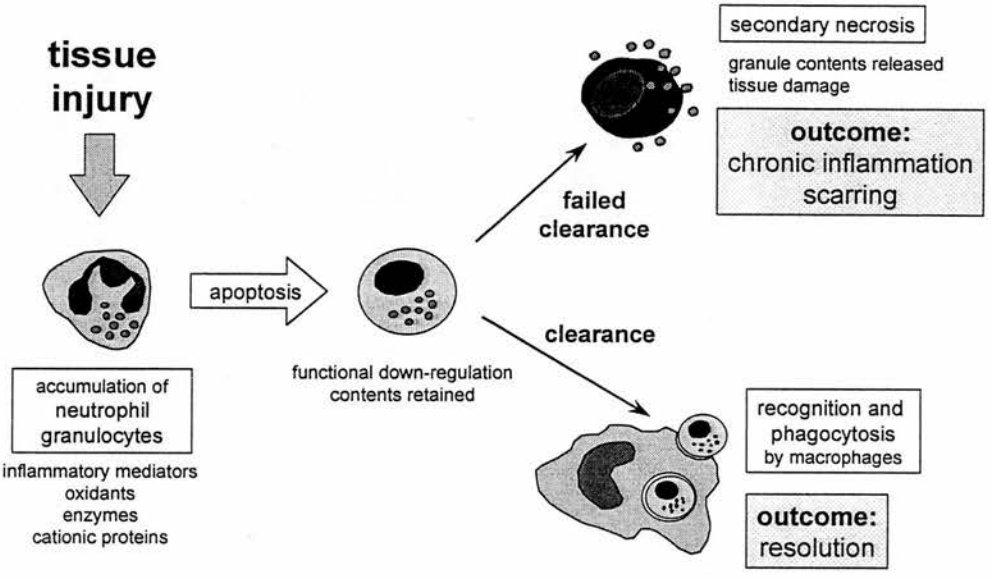


FIGURE 1
Neutrophil apoptosis and phagocytic clearance by macrophages in the resolution of inflammation
See text for details.

The cell biology of phagocytosis

Two distinct phases have been identified during phagocytosis of particles that have been opsonized with immunoglobulin or complement: initial binding to specific receptors on the phagocyte surface followed by internalisation. The dissociation of these two stages has allowed detailed dissection of the pathways at the molecular level. In contrast, few studies have attempted to measure binding of apoptotic cells in the absence of internalisation. Attachment of apoptotic neutrophils may require a temperature- or energy-dependent step since little or no binding to human macrophages is observed at 4°C. In addition, intact cytoskeletal elements (microfilaments and microtubules) are required, as binding of apoptotic cells is not observed when these elements are disrupted (chapter 4). These requirements contrast with those for Fc receptor- and complement receptor (CR)-mediated phagocytosis in which binding still occurs in the absence of intact microfilaments or microtubules (Newman et al., 1991). Microtubular integrity may be particularly important for CR1- and CR3-mediated ingestion, since stabilization of microtubules following elevation of intracellular cGMP has been found to augment phagocytosis. Phagocytosis of apoptotic neutrophils is not affected by treatment with cGMP (Rossi et al., 1998), indicating differential involvement of cytoskeletal components in apoptotic cell clearance when compared with other phagocytic pathways.

In early studies of the mechanisms of phagocytosis via Fc receptors, blockade of surface receptors after particle attachment prevented internalization, suggesting that "zippering" of membrane receptors occurred as lamellaepodia extend around the particle (Griffin et al., 1975). However, for CR-mediated phagocytosis, bound particles appeared to sink into the phagocyte surface, suggesting that internalization may occur via a "modified zippering" mechanism involving formation of a phagocytic pit (Kaplan, 1977). Ultrastructural identification of differences in phagocytic uptake is supported by recent molecular analysis of the recruitment of cytoskeletal elements to nascent phagosomes. Interestingly, during FcR-mediated phagocytosis, cytoskeletal proteins (actin, talin, paxillin, and α–actinin) and tyrosine phosphorylated proteins were enriched near to the phagosome membrane in a diffuse manner and phagocytosis is sensitive to tyrosine kinase inhibitors (Allen and Aderem, 1996). In contrast, during CR-mediated phagocytosis (which is insensitive to tyrosine kinase inhibition) discrete foci of these proteins were distributed over the phagosome surface. These findings suggest intriguing parallels with the recruitment of proteins to nascent phagosomes and the formation of actin-rich podosomes containing talin and tyrosine phosphorylated proteins that have been described during macrophage adherence (Marchisio et al., 1987).

Molecular mechanisms for recognition of apoptotic cells

Clearly, phagocytes must be able to recognise changes on the surface of the apoptotic cell that distinguish it from a healthy viable cell. Many published studies have focused on the molecular mechanisms that may be employed by phagocytes for the clearance of apoptotic cells, and multiple phagocyte surface receptors have been implicated in the recognition process (table II) (Savill et al., 1993; Hart et al., 1996; Devitt et al., 1998).

Surface molecule	Phagocyte	Apoptotic particle
unspecified lectin	mouse peritoneal macrophage (Duvall et al., 1985; Pradhan et al., 1997)	thymocyte, lymphocyte
asialoglycoprotein receptor/ mannose receptor	rat liver cell (Dini et al., 1992), liver endothelial cell (Dini et al., 1995), Kupffer cell (Falasca et al., 1996)	liver cell
a mannose/fucose receptor	human fibroblast (Hall et al., 1994)	neutrophil
$\alpha_V\beta_3$ /thrombospondin ± CD36	human monocyte-derived macrophage (Savill et al., 1990; Savill et al., 1992), fibroblast (Hall et al., 1994), glomerular mesangial cell (Hughes et al., 1997)	neutrophil
	mouse bone marrow-derived macrophage (Fadok et al., 1992), J774 macrophage (Pradhan et al., 1997)	lymphocyte
	human dendritic cell (Rubartelli et al., 1997)	Jurkat cell
phosphatidylserine receptor	mouse peritoneal macrophage, stimulated bone marrow-derived macrophage (Fadok et al., 1992)	lymphocyte
	human PMA-stimulated THP-1 cell (Fadok et al., 1992)	lymphocyte
	rat vascular smooth muscle cell (Bennett et al., 1995)	vascular smooth muscle cell
	rat Sertoli cell (Shiratsuchi et al., 1997)	spermato-genic cell
scavenger receptor	mouse peritoneal macrophage, thymic macrophage (Platt et al., 1996)	thymocyte
61D3 antigen	human monocyte-derived macrophage (Flora and Gregory, 1994)	lymphocyte, neutrophil
	mouse J774 macrophage, peritoneal macrophage (Pradhan et al., 1997)	lymphocyte
ATP binding cassette transporter	mouse peritoneal macrophage (Luciani and Chimini, 1996)	thymocyte
complement receptor CR3/CR4	human retinoic acid-differentiated THP-1 cells (Takizawa et al., 1996)	Jurkat cell

TABLE II
Phagocyte surface receptors that have been implicated in recognition of apoptotic cells

It appears from the published data that in mammals there is significant redundancy in the molecules that are utilised by phagocytes for recognition of apoptotic cells. This is not surprising when it is considered that in the nematode *Caenorhabditis elegans* at least six different genes have been discovered that are involved in the removal of dying cells by phagocytes (Ellis et al., 1991) (figure 2). The reasons for this

redundancy are unclear at present, but phagocytes may employ different mechanisms to recognise cells at different stages of apoptosis, or multiple recognition mechanisms operating in parallel may provide a "fail safe" to ensure that clearance of apoptotic cells occurs effectively.

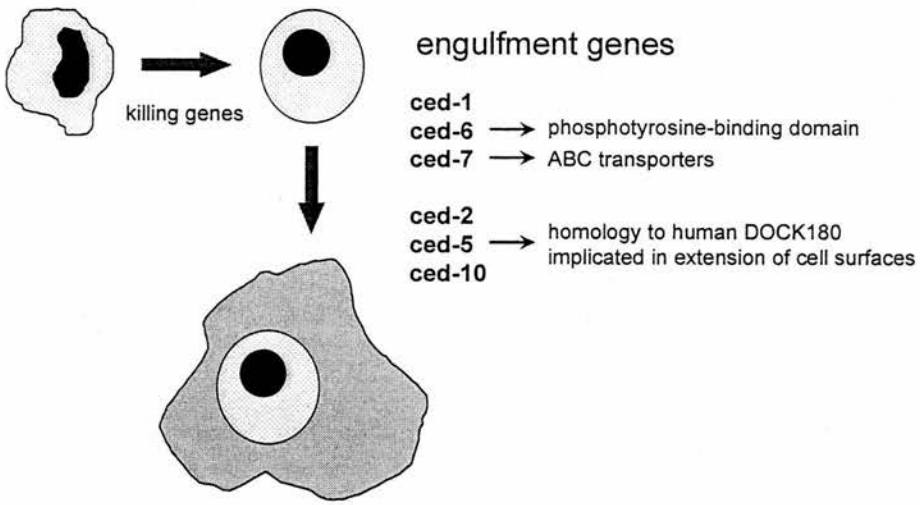


FIGURE 2
Engulfment genes in C. elegans and their mammalian homologs
Important insights into the molecular pathways of apoptosis in mammals have been provided by analysis of genes involved in programmed cell death in the nematode C. elegans. Genes that are involved in the engulfment of cell "corpses" during development of C. elegans have been divided into two functionally redundant groups (ced-1/ced-6/ced-7 and ced-2/ced-5/ced-10). Three genes, ced-5, ced-6, and ced-7, have been cloned and sequenced, providing evidence for the role of similar molecules in the phagocytosis of apoptotic cells in higher animals (arrowed).

Insights from C. *elegans*

During development of C. *elegans* the generation of 959 somatic nuclei is accompanied by deaths of 131 cells, and a number of mutations that affect the processes of cell death and engulfment of dead cells have been identified. In 1983 mutations in two genes were described that specifically blocked the phagocytosis of cells programmed to die during development. The genes were named ced-1 and ced-2. (Hedgecock et al., 1983). Experiments in the late 1980s identified other mutations that prevented normal programmed cell death in C. *elegans*. For example, in ced-3 or ced-4 mutants cells that would normally have died in fact survived and differentiated (Ellis and Horvitz 1986), and the ced-9 gene product appeared to negatively regulate the activity of ced-3 and ced-4 (Ellis et al., 1991). Mutations of one of any six genes, ced-1, 2, 5, 6, 7, or 10, prevented some cell corpses from being engulfed, although in all of these mutants most cells were engulfed normally. In contrast, a major defect in engulfment occurred only in double mutants in which one mutation was in ced-2, 5, or 10, and the other mutation was in ced-1, 6, 7, or 8 (Ellis et al., 1991)(figure 2). This observation suggested that the two sets of genes acted in parallel processes that were partially redundant. It has been recently reported that ced-5 encodes a protein that is similar to

the human protein DOCK180, which has been implicated in the extension of cell surfaces (Wu and Horvitz, 1998). Ced-7 has sequence similarity to the ABC transporters, and has been proposed to translocate molecules that mediate adhesion between the surfaces of the dying and engulfing cells (Wu and Horvitz, 1998a). Cloning and functional characterisation of ced-6 has revealed that the protein product contains a phosphotyrosine binding site, suggesting that ced-6 may be an adapter molecule acting in a signal transduction pathway that mediates recognition and engulfment of apoptotic cells (Liu and Hengartner, 1998). Mammalian homologs of the other ced gene products remain to be elucidated.

Apoptotic cell ligands

In contrast with phagocyte surface molecules, the changes on the surface of the apoptotic target cell that are responsible for phagocyte recognition have been poorly characterised. In the erythrocyte (which has an average lifespan of 120 days in the healthy adult human) post-translational modification of the anion transport protein band 3 may lead to binding of endogenous IgG autoantibodies and subsequent Fc receptor-mediated phagocytosis (Kay, 1981; Kay et al., 1996). In parallel, non-enzymatic glycosylation of surface proteins to form advanced glycosylation end products (AGE) may allow engagement of specific receptors (e.g., the immunoglobulin superfamily member RAGE) that lead to cell clearance. However, the kinetics of appearance of AGE on senescent cells is likely to be too slow to serve as a signal for apoptotic cell recognition by phagocytes. In addition, the patterns of inhibition of macrophage phagocytosis of apoptotic neutrophils using polyanionic ligands indicated that AGE and AGE receptors were unlikely to participate (Savill et al., 1989).

The anionic phospholipid phosphatidylserine (PS) has been the subject of a number of published studies (Verhoven et al., 1995). In healthy cells PS is confined by a phospholipid translocase to the inner half of the plasma membrane bilayer, but is specifically relocated to the outer surface of the membrane during apoptosis in many cell types, including neutrophils (Homburg et al., 1995). PS exposure has been implicated in the recognition of apoptotic leukocytes by mouse inflammatory macrophages, but not human macrophages, in vitro (Fadok et al., 1992). Recently it has been reported that complement components C1q and iC3b may bind to the surface of certain apoptotic cell types, which may opsonize them for phagocytic removal via C1q receptors or CR3/CR4 (Takizawa et al., 1996; Korb and Ahearn, 1997). However, CR3 was apparently not involved in the recognition of apoptotic neutrophils by human monocyte-derived macrophages (Savill et al., 1989).

Other molecular changes in the membrane of apoptotic cells have been studied using monoclonal antibodies and lectins as probes. Early studies indicated that cellular microelectrophoretic mobility was reduced on apoptotic mouse thymocytes, suggestive of changes in the net negative charge of cell membranes (Morris et al., 1984). Reduced binding of lectins and antibodies observed in flow cytometric analysis corresponded closely with the degree of reduction in membrane surface area following apoptosis (Morris et al., 1984). It has been reported that high levels of mannose, N-acetylgalactosamine, and galactose can be detected on apoptotic human peripheral blood lymphocytes using fluoresceinated lectins (Dini et al., 1992). However, flow cytometric analysis of binding of a panel of conjugated lectins to

apoptotic neutrophils suggests that exposure of these carbohydrate moieties may not be a general feature of apoptosis on all cell types (chapter 5).

There is also evidence that specific changes in surface protein expression accompany apoptosis (Dransfield et al., 1994; Dransfield et al., 1995) implying that mechanisms for maintenance of receptor numbers are compromised during programmed cell death. Neutrophil apoptosis is associated with a marked down-regulation of FcγRIII (CD16) (Dransfield et al., 1994), leukosialin (CD43), and L-selectin (CD62L) (Dransfield et al., 1995), which may provide a mechanism for functionally isolating apoptotic neutrophils from potentially pro-inflammatory signals. Since these receptors are shed by proteolytic mechanisms during activation, one possibility is that there is activation of surface associated proteases during apoptosis, or alternatively, release of functional constraint upon protease activity by as yet undefined mechanisms. Whether proteolytic shedding of surface receptors is a fundamental feature of apoptosis in different cell types remains to be determined. Screening of a large panel of antibodies revealed that blockade of certain epitopes of ICAM-3 (CD50) on apoptotic B cells was found to inhibit their phagocytosis by macrophages (Gregory et al., 1998). Since ICAM-3 is also expressed on all non-apoptotic leukocytes, additional uncharacterised molecular alterations (e.g., altered glycosylation) may be required for phagocyte recognition. Apart from the identification of PS exposure as a molecular event common to all cells undergoing apoptosis, the evidence for a unique signal that determines the capacity for recognition and subsequent phagocytosis of apoptotic cells remains poor. Moreover, given the huge variety of macrophage surface molecules that have been proposed to be involved in the recognition of apoptotic cells it is very likely that there are many additional undefined molecular changes on the surface of apoptotic cells that signal recognition under different circumstances.

Regulation of phagocytosis of apoptotic cells

If the rate of cell death by apoptosis is such that macrophage clearance capacity is exceeded, apoptotic cells may progress to secondary necrosis, resulting in release of harmful cellular contents and damage to the surrounding tissue (figure 1). For example, treatment of mice with Fas antibody triggered a massive wave of apoptosis in the liver, and the animals developed extensive hepatic necrosis and died (Ogasawara et al., 1993). Similarly, induction of apoptosis in the rat lung may lead to pulmonary fibrosis (Hagimoto et al., 1997). Presumably, in these situations the hepatic and pulmonary macrophages respectively were unable to clear the load of apoptotic cells. Similar situations could potentially arise if neutrophil apoptosis was to be deliberately induced as part of a therapeutic strategy for inflammatory disease.

In order that the load of apoptotic neutrophils at an inflammatory site is matched by appropriate clearance activity, macrophage capacity for apoptotic cell phagocytosis is likely to be closely regulated. Macrophage phagocytic capacity may be influenced by local soluble mediators such as cytokines (Ren et al., 1995) and prostaglandins (Rossi et al., 1998), or by glucocorticoid hormones (Liu et al., 1999). In addition, interaction of surface adhesion molecules with neighbouring cells and extracellular matrix components may profoundly influence many aspects of cellular behaviour, including phagocytosis (Brown, 1986).

Adhesion molecules regulate leukocyte function

Engagement of leukocyte adhesion molecules by ligands in the extracellular matrix (ECM) and on neighbouring cells leads to assembly of cytoskeletal components and activation of intracellular signal transduction cascades (Giancotti, 1997). In this way, adhesion molecules act as sensors of the external milieu so that in concert with soluble chemical mediators the behaviour of extravasated inflammatory cells can be coordinated (Nathan and Sporn, 1991). There are several lines of evidence to suggest that macrophage capacity for phagocytosis of apoptotic cells may by modulated by the adhesive state of the macrophage:

1) Inflammatory responses to tissue injury are associated with sequential changes in the local composition of the ECM (Gailit and Clark, 1994). Furthermore, macrophages are able to synthesise and lay down their own matrix (including fibronectin) during in vitro culture (Alitalo et al., 1980), and serum contains a variety of matrix molecules including fibronectin and thrombospondin. Thus, in vitro culture conditions may influence macrophage capacity for phagocytosis of apoptotic cells.

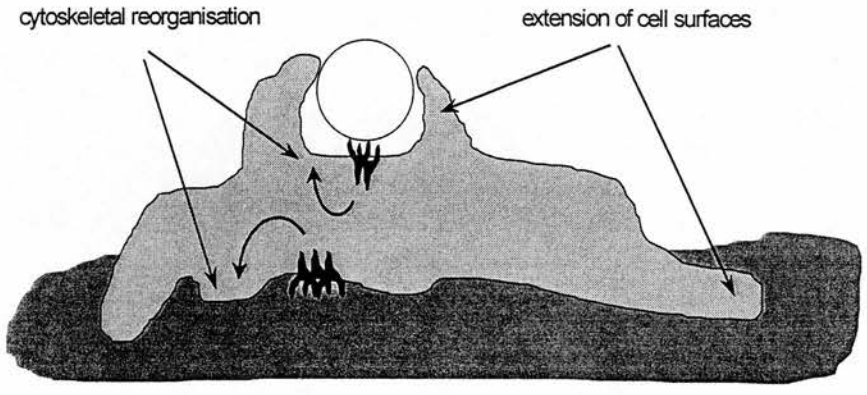
2) It has been shown that phagocytosis of opsonized particles in vitro is increased when macrophages are allowed to adhere to components of the ECM such as fibronectin (Wright et al., 1983; Brown, 1986; Newman and Tucci, 1990).

3) Elevation of intracellular cyclic AMP in macrophages, which may follow binding of soluble mediators (such as prostaglandins) to adenylate cyclase-linked receptors, rapidly and specifically inhibits recognition of apoptotic neutrophils, and is associated with changes in the localisation of the cytoskeletal proteins actin and talin within the macrophages (Rossi et al., 1998).

4) Recent studies of C. elegans mutants that display defective phagocytic removal of cellular corpses have characterised a number of genes involved in this process that may give important clues about the nature of mammalian apoptotic cell recognition pathways. For example, the ced-6 gene product contains sequences similar to mammalian phosphotyrosine binding domains, and the protein encoded for by ced-5 shows homology to the human CRK-binding protein DOCK-180 that has been implicated in cytoskeletal function and extension of cell surfaces. Remodelling of the cellular cytoskeleton is an integral component of a variety of related cellular processes, including adhesion, migration, and phagocytosis (Allen and Aderem, 1996a)(figure 3).

A role for the adhesive state of the macrophage in modulating apoptotic cell clearance has been confirmed in vitro by recent findings that adhesion of human macrophages to the matrix molecules fibronectin, vitronectin, or collagen IV exhibited significantly enhanced their capacity for phagocytosis of apoptotic neutrophils (McCutcheon et al., 1998). Further analysis using monoclonal antibodies and recombinant fibronectin fragments revealed that β1 integrin mediated adhesion to fibronectin was partly responsible for the augmented phagocytosis, but that additional (and as yet unidentified) macrophage receptors also played a role.

FIGURE 3
Cell adhesion, migration, and phagocytosis are similar processes
Ligation of phagocyte surface receptors by ligands either in the extracellular matrix or on the target



particle induces intracellular signalling pathways and cytoskeletal reorganisation, resulting in extension of cell surfaces.

CD44 is an adhesion receptor that has been implicated in adhesion to a variety of matrix components including fibronectin, and may be associated with cytoskeletal proteins and intracellular signalling pathways (Lesley et al., 1993a). Furthermore, CD44 has been proposed to play a role in the regulation of macrophage function in inflammation (Noble et al., 1993; Mikecz et al., 1995). The aim of this thesis was to investigate the role of macrophage CD44 in phagocytosis of apoptotic neutrophils, a key process in the resolution of inflammation.

Structure and Function of CD44

The CD44 gene in humans consists of 50-60 kilobase pairs located on the short arm of chromosome 11. Some of the 20 or more exons are constitutively transcribed, but a sequence of at least 10 exons (exons 6a-14) undergoes variable splicing, giving rise to an additional peptide sequence between amino acids 202 and 203 in the membrane proximal domain of the CD44 protein (Screaton et al., 1992; Cooper and Dougherty, 1995) (figure 4). In practice only relatively few variant isoforms of CD44 (CD44v isoforms) containing certain combinations of variably spliced CD44 exons have been found to be expressed in human cells. The most prevalent form of CD44, termed CD44S ("standard") or CD44H ("haematopoietic"), contains no additionally spliced exon products. CD44S is a single chain type I transmembrane protein containing 341 amino acids, giving a predicted molecular weight (MW) of 40 kD. However, extensive post-translational glycosylation results in CD44S having an apparent MW on SDS-PAGE of 80-100 kD (Camp et al., 1991).

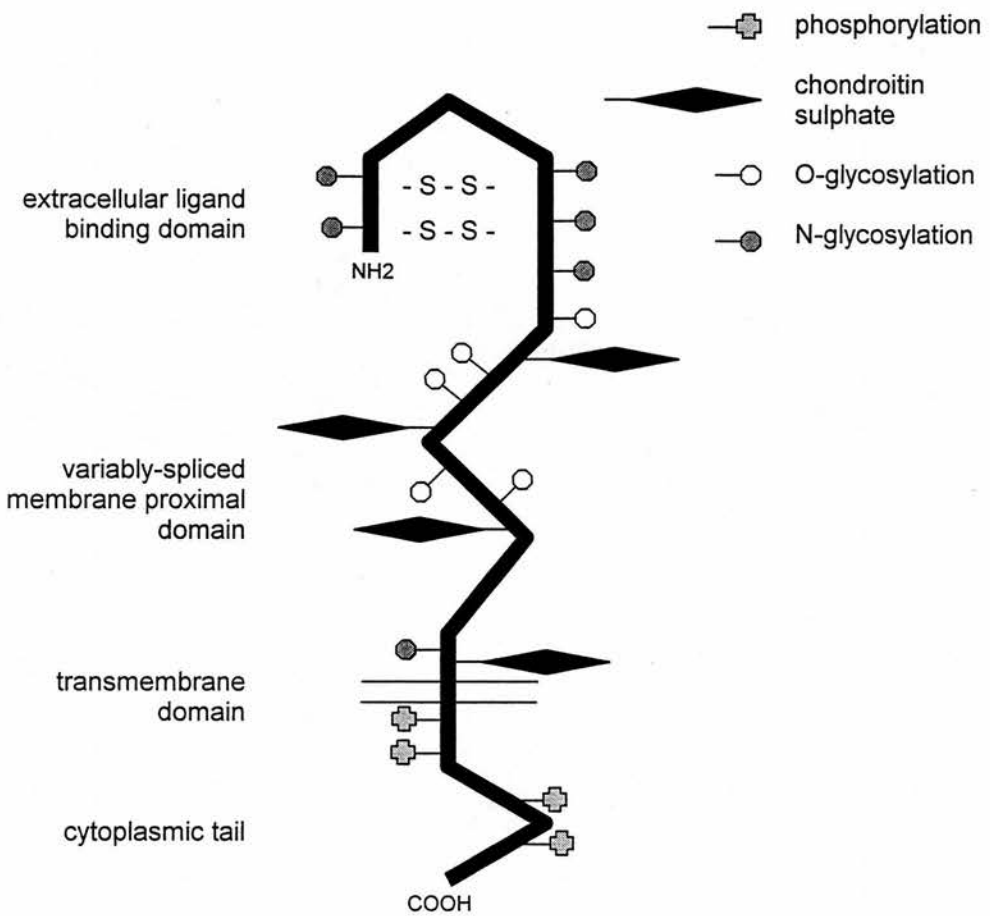


FIGURE 4
Diagrammatic representation of CD44
CD44 comprises a large extracellular ligand-binding domain, an alternatively spliced membrane proximal domain containing sites for linkage of large sugars, a highly conserved transmembrane sequence, and a short cytoplasmic tail containing phosphorylation sites.

Clues about the possible function of CD44 were generated when monoclonal antibodies against three separately identified antigens were found to recognise the same molecule (Picker et al., 1989). The Hermes lymphocyte adhesion antigen had been proposed to specifically mediate lymphocyte adhesion to high endothelial cells lining post-capillary venules in lymphoid tissue (Jalkanen et al., 1986). Expression of Pgp-1, a polymorphic murine glycoprotein, was up-regulated on prothymocytes and activated T lymphocytes. Antibodies in the CDw44 cluster recognized a broadly distributed human glycoprotein related to the Lutherian blood group antigen that was a marker for medullary thymocytes. Following the discovery that these proteins were identical, expression cloning of CD44 revealed that the amino terminal portion of the protein bore remarkable homology with cartilage link proteins (Goldstein et al., 1989; Stamenkovic et al., 1989). Interaction between hyaluronan and hyaluronan-binding proteins is crucial for the formation and stability of the extracellular matrix as well as many aspects of cell behaviour during embryological development, morphogenesis, tumour biology, and inflammation (Knudson and Knudson, 1993). In cartilage the link proteins stabilise large aggregates of proteoglycan core protein and the linear sugar hyaluronan (also known as hyaluronic acid or hyaluronate) by binding either the proteoglycan monomer or hyaluronan. These interactions are mediated by a common protein domain called the link module (previously known as the proteoglycan tandem repeat). The link module consists of approximately 100 amino acids and contains four disulphide-bonded cysteine residues. Link modules are present in

matrix proteins such as aggrecan, link protein, and the arthritis-associated protein TNF-stimulated gene-6 (TSG-6). Recent analysis of the solution structure of the link module has shown that it contains two alpha helices and two antiparallel beta sheets. Interestingly, the link module bears structural similarity to the C-type lectin domain of mannose binding protein (Kohda et al., 1996) .

Interest in CD44 isoforms was initiated by the observation that their expression by certain tumours was associated with aggressive metastatic potential (Lesley et al., 1993a). The significance of variably spliced isoforms of CD44 in other cell types is less clear, but attachment of large O-linked glycosaminoglycans to specific residues in the additional membrane proximal peptide sequence may be responsible for their altered ligand binding specificity compared with CD44S. For example, CD44R1, which is expressed by certain endothelial cells, contains exons v3-v10 and binds less well than CD44S to hyaluronan in vitro (Dougherty et al., 1994).

CD44 is expressed by many cell types, including leukocytes, erythrocytes, fibroblasts, endothelial and epithelial cells, and a variety of tumour cells (Lesley et al., 1993a). Unusually for a cell surface receptor, CD44 seems to have broad ligand specificity and has been proposed to bind the ECM molecules hyaluronan (Aruffo et al., 1990; Culty et al., 1990), fibronectin (Jalkanen and Jalkanen, 1992), collagen (Faassen et al., 1992), and fibrin (Henke et al., 1996; Svee et al., 1996). In addition, CD44 has been proposed to bind to the proteoglycan serglycin found in lymphocyte and mast cell granules (Toyama-Sorimachi et al., 1995), the extracellular phosphoprotein osteopontin (Weber et al., 1996), and to CD44 molecules on neighbouring cells (Droll et al., 1995). The interaction of cell surface CD44 with hyaluronan is thought to be important in a variety of cell adhesion-related phenomena (table III), but the functional relevance of CD44 binding to other putative ligands is poorly understood at present.

CD44-hyaluronan interactions

Hyaluronan is a large linear sugar composed of alternating N-acetyl glucosamine and D-glucuronic acid subunits (Laurent et al., 1996). It is not associated with a core protein, and depending on the length of the sugar chain its molecular weight varies from 100000 to 6 million Daltons. Hyaluronan is synthesised by fibroblasts, macrophages, and other cell types, and is a constitutive component of extracellular matrix, being especially prevalent in articular cartilage. Following tissue injury, the local concentration of hyaluronan rises rapidly to reach a peak at 48 hours (Gailit and Clark, 1994), before declining again as it is endocytosed by macrophages (Fraser et al., 1997). In vivo a variety of inflammatory diseases have been associated with tissue accumulation of hyaluronan (Hallgren et al., 1985; Hallgren et al., 1989; Nettelbladt et al., 1989). CD44 is the principal cell surface receptor for hyaluronan (Aruffo et al., 1990).

Process	Examples
Hyaluronan-dependent cell adhesion	Rolling adhesion of lymphocytes on endothelium (DeGrendele et al., 1996)
	Homing of lymphocytes to lymphoid organs
	Homotypic aggregation of macrophages and other cell types (Green et al., 1988; Culty et al., 1990)
	Adhesion of lymphocytes to white matter (Aho et al., 1994) and astrocytes (Haegel et al., 1993) in the central nervous system
	Adhesion of fibroblasts to keratinocytes (Milstone et al., 1994)
	Tumour formation (Bartolazzi et al., 1995) and metastases (Price et al., 1996)
Stimulation of β2-integrin mediated adhesion	Homotypic aggregation of T lymphocytes (Koopman et al., 1990) (Vermot-Desroches et al., 1995)
Cytoskeletal changes	CD44 is linked to ERM proteins (Tsukita et al., 1994) in BHK cells
	Morphological changes in human PB T lymphocytes (Kelleher et al., 1995)
	B cell spreading (Santos-Argumedo et al., 1997)
Monocyte cytokine production	M-CSF (Gruber et al., 1992)
	IGF-1 (Noble et al., 1993)
	chemokines (McKee et al., 1996)
	TGF-β and IL-1 (Rameshwar et al., 1996)
	TNF-α (Zembala et al., 1994)
Intracellular signalling	Elevated cyclic AMP (Rothman et al., 1993) in T lymphocytes
	Activation of $p56^{lck}$ tyrosine kinases in T lymphocytes (Taher et al., 1996)
	Tyrosine phosphorylation of various substrates in NK cells (Galandrini et al., 1996)
Cellular activation	Neutrophil cytotoxicity (Pericle et al., 1996)
	T lymphocytes proliferation (Pierres et al., 1992)
	NK cell activation (Tan et al., 1993)
	Dendritic cell/ Langerhans cell activation (Weiss et al., 1997)
Apoptosis	Inhibition of CD3- and dexamethasone-stimulated lymphocyte apoptosis (Cannarile et al., 1995)
	Induction of fibroblast apoptosis (Henke et al., 1996)

TABLE III
Pathophysiological roles of CD44

Binding of hyaluronan to CD44 may be regulated by the presence of additional peptide sequences in the membrane-proximal domain of CD44 (Dougherty et al., 1994), variable glycosylation of the extracellular domain (Bennett et al., 1995; Katoh et al., 1995; Bartolazzi et al., 1996; Lesley et al., 1995; Takahashi et al., 1996), phosphorylation of serine residues in the cytoplasmic tail (Isacke, 1994; Peck and Isacke, 1996), and by intracellular signals that induce CD44 dimerization via the transmembrane domain (Liu and Sy, 1997).

Macrophage CD44: An Important Adhesion Molecule in Inflammation

Freshly isolated peripheral blood monocytes express CD44S strongly (MacKay et al., 1994), and differentiation of monocytes into macrophages during in vitro culture is associated with increased expression of variant isoforms while CD44S expression persists at high levels (Culty et al., 1994; Levesque and Haynes, 1996). Elevated local concentrations of CD44 ligands (such as hyaluronan and fibronectin) that follow tissue injury are likely to be important mediators of macrophage function as the inflammatory response progresses. Several observations support the suggestion that macrophage CD44 is important in inflammatory processes:

1) Macrophage expression of certain CD44 variant isoforms (CD44v6 and CD44v9) is increased at sites of chronic inflammation (sarcoidosis) (Levesque and Haynes, 1996), and expression of the same isoforms by monocyte-like THP-1 cells can be upregulated following exposure to the inflammatory cytokines TNF-α or IFN-γ (MacKay et al., 1994).

2) Cross-linking of monocyte or macrophage CD44 with antibodies or hyaluronan induced production pro-inflammatory or pro-fibrotic cytokines in vitro (Webb et al., 1990; Gruber et al., 1992; Noble et al., 1993). Activation of the nuclear transcription factor NF-κB (Noble et al., 1996) leading to increased transcription of cytokine mRNA (McKee et al., 1996) is likely to be the mechanism of augmented cytokine production.

3) In vivo, systemic administration of CD44 monoclonal antibodies reduced the severity of joint inflammation in mice with experimental arthritis (Mikecz et al., 1995; Verdrengh et al., 1995).

These studies suggest that CD44 may play a number of different roles, both pro-inflammatory and anti-inflammatory, depending on the experimental system used. However, these observations support a role for CD44-mediated macrophage-ECM interactions in regulating the outcome of inflammation.

AIMS

The aim of this thesis is to investigate the role of macrophage CD44 in the phagocytosis of apoptotic neutrophils in vitro. Initially, expression of CD44 by human monocyte-derived macrophages will be characterised by indirect immunofluorescence and flow cytometry, and by immunoprecipitation and Western blotting. Next, the role of CD44 in macrophage phagocytosis of apoptotic cells will be investigated in functional studies by adding monoclonal antibodies to an in vitro phagocytic assay. I then plan to further investigate the molecular events at the macrophage surface associated with CD44-modulated phagocytosis, and assess macrophage response to apoptotic cell phagocytosis in terms of cytokine production. Inititial characterisation of the intracellular signalling events that follow ligation of macrophage CD44 will be described. Finally, I will analyse some of the changes on the surface of the apoptotic neutrophil using a novel monoclonal antibody and a panel of lectins.

CHAPTER 2
METHODS

Antibodies and other reagents

All biochemicals were purchased from Sigma Chemical Co., Poole, UK. Cell culture materials were from Life Technologies, Paisley, Scotland, UK, Percoll was from Pharmacia, St. Albans, UK, and cell culture plastic was from Falcon Plastics, provided by A.& J. Beverage, Edinburgh, UK.

Mouse monoclonal antibodies (IgG_1 unless specified) recognising all isoforms of CD44 were 5A4 (Dougherty et al., 1994; Droll et al., 1995) and F10.44.2 (IgG_{2a}; provided by Dr. S. Howie, Department of Pathology, Edinburgh University, UK). Other CD44 antibodies were 7F4 (against a hyaluronan binding-associated epitope (Dougherty et al., 1994; Droll et al., 1995)); 3G5 (IgG_{2b}; CD44v3; R&D Systems, Abingdon, UK); VFF-17 (IgG_{2b}; CD44v7-8; Bender Medsystems, UK); and 2G1 (CD44v10 (Droll et al., 1995)).

FITC-conjugated 3G8 was then prepared as described previously (Dransfield et al., 1994) and used at a final concentration of 2 μg/ml.

Monoclonal antibody Bob93 (IgG isotype) was prepared by Dr J. Ross, Edinburgh University Department of Surgery, by fusion of splenocytes from a BALB/c mouse immunised with the human myelomonocytic cell line THP-1 (obtained from the E.C.A.C.C., Porton Down, U.K.) with Sp2/0'-Ag14 (E.A.C.C.) non-secreting myeloma cell line. Fusion products secreting Ig were then tested further in flow cytometry for reactivity with apoptotic, but not freshly isolated neutrophils and sub-cloned twice prior to further analysis. No previously characterised antibodies that we have tested showed similar reactivity to antibody Bob93 suggesting that the antigen recognised by BOB93 may be unique.

Other antibodies were sourced as described in table I.

Preparation of Fab' and $F(ab')_2$ antibody fragments

Fab' and $F(ab')_2$ fragments of purified CD44 antibody 5A4 were prepared using previously described methods (Dransfield et al., 1992). Monoclonal antibody 5A4 was purified from hybridoma supernatant on a protein A-agarose column. Purified antibody was subjected to pepsin digestion (1% wt/wt) to prepare $F(ab')_2$ fragments, followed by reduction with 0.03M iodoacetamide for one hour at room temperature to prepare Fab' fragments. Intact antibody and synthesised fragments were run on a non-reducing 10% polyacrylamide-SDS gel, blotted onto nitrocellulose, and visualised with 1:1000 anti-mouse Ig-HRP (Dako) and 0.5 mg/ml diaminobenzidine containing 0.03% H_20_2 in PBS. Purified Fab' and $F(ab')_2$ fragments were free of intact antibody as determined by gel electrophoresis and immunoblotting.

Antibody	Source
Rabbit anti-human IgG	Dako Ltd, High Wycombe, UK
FITC-conjugated F(ab')$_2$ goat anti-mouse Ig	Dako
PE-conjugated F(ab')$_2$ goat anti-mouse Ig	Dako
HRP-conjugated anti-mouse Ig	Dako
FITC-donkey anti-rabbit Ig	SAPU
Wac70 (CD11a; IgG2a)	Dr J. Ross, Edinburgh University
MC2 (CD15; mouse IgM)	Dr. M. Kerr, Dundee
3G8 (CD16; supernatant)	Dr. J. Unkeless, New York
TS1-18 (CD18)	ATCC
IV3 (CD32)	Medarex, N.Y.
E11 (CD35; ascites)	Dr. N. Hogg, ICRF
SMϕ (CD36; IgM)	Dr. N. Hogg, ICRF
CLB-IVC7 (CD36)	Eurogenetics U.K. Ltd
ezrin	Affiniti
5A4 (pan-CD44; supernatant)	Dougherty et al., 1994; Droll et al., 1995
F10.44.2 (pan-CD44)	Dr. S. Howie, Edinburgh University
7F4 (CD44 HA-binding epitope)	Dougherty et al., 1994; Droll et al., 1995
3G5 (CD44v3)	R&D Systems
Lck	Affiniti
VFF-17 (CD44v7-8)	Bender Medsystems, UK
2G1 (CD44v10)	Droll et al., 1995
23C6 (CD51/61)	Dr. M. Horton, ICRF, London
W6/32 (MHC class I; mouse IgG2a)	ECACC
MOPC31C (mouse plasmacytoma; supernatant)	ECACC
61D3 (ascites)	Dr C. Gregory, Birmingham University
paxillin	Affiniti
RC20 (phosphotyrosine)	Affiniti
NC1 (anti-IgM)	Coulter, UK
NC2 (anti-IgG1)	Coulter, UK
NC3 (anti-IgG2a)	Coulter, UK
Bob93 (supernatant)	Dr J. Ross, Edinburgh University

TABLE I

Antibodies used in this thesis

All antibodies are purified mouse IgG_1 unless stated otherwise. All antibodies were used at concentrations that saturated binding as assessed by flow cytometric analysis with an EPICS™ Profile II cytometer (Coulter Electronics, Hileagh, FL). ATCC, American type culture collection; ECACC, European collection of animal cell cultures; SAPU, Scottish antibody production unit.

Cell Isolation

Mononuclear cells and neutrophils were isolated from peripheral blood by dextran sedimentation and discontinuous Percoll gradient centrifugation as described (Ackerman and Douglas, 1978; Partridge and Dransfield, 1993; Dransfield et al., 1994). Neutrophils were cultured at $4x10^6$/ml in Iscove's modification of Dulbecco's modified Eagle's medium (Iscove's medium) containing 10% autologous serum at 37°C in a 95% air/5% CO_2 atmosphere for 20-24 hours, during which time a proportion of the cells underwent apoptosis (Savill et al., 1989). Apoptosis was assessed by nuclear morphology under light microscopy, loss of CD16 expression, or gain of annexin V binding in flow cytometry. Mononuclear cells were suspended at $4x10^6$/ml in IDMEM and allowed to adhere to 48 well plates (Costar; 0.5 ml per well) during incubation at 37°C for 1 hour. Non-adherent cells (predominantly lymphocytes) were collected and the adherent cells (>90% monocytes) washed twice in HBSS containing Ca^{2+}/Mg^{2+}. Monocytes/macrophages were then cultured for five to seven days in Iscove's medium containing 10%

autologous serum, and the medium was changed on the third day. For some experiments, macrophages were cultured adherent to 13 mm glass coverslips in 24 well plates.

For flow cytometric analysis macrophages were detached from cell culture plates by vigorous pipetting following incubation on ice with PBS for 30 minutes.

THP-1 and J774 cells were cultured in RPMI1640 containing 10% FCS and were washed three times with PBS before use.

Immunoglobulin G-opsonized erythrocytes (EIgG) were prepared by incubating washed erythrocytes from human blood with a 1:4000 dilution of polyclonal rabbit anti-human erythrocyte antibody for 1 hour at 37 °C. In order to maximise the chance of observing any augmentation of phagocytosis, a concentration of opsonizing antibody was chosen that, in preliminary experiments, resulted in phagocytosis of EIgG by approximately 30% of macrophages. Previous studies have shown that monocyte phagocytosis of E(IgG) can be augmented using similarly prepared cells (Pommier et al., 1983). The E(IgG) were washed twice in PBS and suspended in IDMEM at $4x10^6$/ml.

Induction of lymphocyte apoptosis

Apoptosis was induced in freshly isolated lymphocytes by γ-irradiation (1250 rads) and culture for a further 24 hours in RPMI 1640 with 10% FCS. For staining of cells using acridine orange, cells were incubated with 5μg of acridine orange in PBS (stock 5mg/ml in dimethyl formamide) and examined using an Olympus BH-2 microscope fitted with a fluorescent lamp attachment.

Flow cytometry

Indirect immunofluorescence was used to assess antibody binding to leukocytes using FITC- or PE-conjugated $F(ab')_2$ goat anti-mouse Ig. Single and dual colour flow cytometric analysis was performed as previously described (Dransfield et al., 1994; Dransfield et al., 1995). Fluorescence of monocytes and lymphocytes were assessed by gating peripheral blood mononuclear cells on the basis of well defined forward and side scatter profiles. For double labelling experiments, phycoerythrin-conjugated $F(ab')_2$ goat anti-mouse immunoglobulin was used as a second layer and an additional incubation with FITC-labelled 3G8 (anti-CD16) was performed prior to washing and flow cytometric analysis.

Enzyme treatment of the cell surface

Human monocyte-derived macrophages adherent to 48 well plates were incubated for 20 minutes at room temperature with antibody 5A4 (1:5 supernatant) or medium alone, washed, and then incubated for 30 minutes at 37°C with 100 μg/ml trypsin type IX (from porcine pancreas), 100 μg/ml pronase, 100 μg/ml proteinase K, or 0.1 U/ml phosphatidylinositol specific phospholipase C (PI-PLC). The cells were washed thoroughly and phagocytosis of apoptotic neutrophils was then assessed.

Twenty-hour aged human peripheral blood neutrophils were incubated for 30 minutes at 37°C with 100 μg/ml trypsin type IX (from porcine pancreas), 100 μg/ml proteinase K, or 0.1 U/ml Vibrio cholerae neuraminidase. After washing, the cells were then incubated with the macrophage monolayer and phagocytosis of apoptotic neutrophils was assessed.

Treatment with neuraminidase completely abolished binding of monoclonal antibody sLex to neutrophil sialyl-Lewis^x, and CD14 expression was reduced by 50% as determined by binding of the antibody UCHM1.

Immunoprecipitation of CD44

Human monocyte-derived macrophages cultured on six well plates (Costar; approximately 10^6 cells per well) were surface labelled with biotin by incubation for 60 minutes at room temperature with 0.1 mg/ml NHS-LC-biotin (Pierce) in PBS containing divalent cations (2 ml per well). The macrophage monolayers were washed and then lysed with 200 µl/well ($5x10^6$ cells per ml of lysate) of Tris-buffered saline (pH 7.5) containing 1% NP-40 and protease inhibitors (Boehringer protease inhibitor cocktail) for 20 minutes on ice. The lysates were spun at 10^5g to remove nuclear and cytoskeletal material and the supernatants incubated with monoclonal antibodies MOPC (control), W6/32 (MHC class I), or 5A4 (CD44; final dilution 1:2 supernatant) for 60 minutes on ice. 20 µl of anti-mouse Ig-agarose (Sigma) was then added to each tube (60 µl of a 1:3 suspension in lysis buffer) and agitated for 60 minutes at 4°C. The pellets were washed five times with lysis buffer and then boiled in 4x reducing Laemmli SDS sample buffer. Immunoprecipitates (20 µl per lane) were run on a reducing 10% polyacrylamide gel and electroblotted onto nitrocellulose membrane (Amersham). The membrane was blocked with Tris-buffered saline pH 7.5 containing 0.1% Tween 20 for 16 hours at 4°C. Products were visualised by incubation with 1:5000 streptavidin-HRP (Dako) and development using enhanced chemiluminescence (Amersham).

Macrophage phagocytosis assay

Macrophage phagocytosis of apoptotic neutrophils was assessed using minor modifications of a previously described serum-free phagocytosis assay (Newman et al., 1982; Savill et al., 1989). Adherent macrophages (<10% contaminating lymphocytes as assessed by morphology after staining with Diff-Quik^{TM}) were washed with Iscove's medium and incubated with CD44 antibody 5A4 (1:5 supernatant) in Iscove's medium for 20 minutes at 20°C. Macrophages were washed again with Iscove's medium and $4x10^6$ aged neutrophils (>50% apoptotic and >98% membrane impermeable as assessed by exclusion of trypan blue) in 0.5 ml Iscove's medium were added to each well. After 30 minutes' incubation at 37°C the wells were washed vigorously with ice-cold PBS without cations, fixed in 2.5% glutaraldehyde, and stained for myeloperoxidase (MPO) with 0.1 mg/ml dimethoxybenzidine and 0.03% (v/v) hydrogen peroxide in PBS. The percentage of macrophages (MPO-negative) that had phagocytosed one or more apoptotic neutrophils (MPO-positive) was quantified by examination with an inverted microscope of at least five fields (minimum 400 cells), and recorded as the mean percent phagocytosis of duplicate or triplicate wells. The phagocytic nature of the interaction was confirmed by examining cytocentrifuge preparations of macrophages detached with trypsin-EDTA (Gibco) and by transmission electron microscopy.

Assessment of phagocytosis of apoptotic lymphocytes was performed in a similar manner, but with some modifications to the method in order to accurately identify phagocytosed cells. Irradiated lymphocytes ($8x10^6$/ml in Iscove's medium; >90% pure, 20-40% apoptotic (as assessed by acridine orange staining

and examination of nuclear morphology, and surface labelling with FITC-annexin V in flow cytometry), and >90% trypan blue negative) were added to macrophages and incubation and washing were performed as described for neutrophils. Macrophages were detached by incubation with 0.05% trypsin-0.02% EDTA for 10 minutes at 37°C, and 100 µl the cell suspension from each well was cytocentrifuged onto a separate glass slide. Examination by light microscopy of slides fixed in methanol and stained with Diff-Quik™ permitted identification of apoptotic lymphocytes within macrophage phagocytic vacuoles, and the percentage of macrophages that had phagocytosed apoptotic lymphocytes and the average number of apoptotic lymphocytes ingested per phagocytosing macrophage were determined (minimum 300 macrophages counted per slide). This counting method was validated with the macrophage/apoptotic neutrophil interaction, both with and without CD44 antibody treatment, which gave similar results to the standard 'in well' counting method described above.

Assessment of phagocytosis of EIgG was performed as described for aged neutrophils except that $4x10^6$ E(IgG) in 0.5 ml Iscove's medium were added to the macrophage monolayer. After incubation and washing, non-phagocytosed erythrocytes were lysed with a ten second application of distilled water before immediate fixation in 2.5% glutaraldehyde. For assessment of zymosan phagocytosis macrophages cultured on glass coverslips were treated with CD44 antibody and washed as described above. Zymosan A was boiled for 15 minutes in PBS, washed twice in PBS, and resuspended in Iscove's medium at 2 mg/ml. A 50µl droplet of zymosan suspension was added to each coverslip and incubated at 37°C for 30 minutes before vigorous washing with cold HBSS and fixation in methanol. Zymosan was stained with periodic acid (5 minutes) and Schiff reagent (10 minutes) and macrophages counterstained with Diff-Quik™. The percentage of macrophages that had phagocytosed zymosan particles was determined as described above.

Role of cytoskeletal elements

Human monocyte-derived macrophages in 48 well plates were washed and incubated for 15 minutes at room temperature with Iscove's medium containing no inhibitor, cytochalasin B 0.1 µg/ml, cytochalasin B 5 µg/ml, colchicine 10 µg/ml, or nocodazole 2.5 µg/ml. In the continued presence of the inhibitors the macrophages were stimulated with CD44 antibody 5A4 (1:5 supernatant) for a further 15 minutes, and then phagocytosis of apoptotic neutrophils was assessed again in the presence of the same inhibitors.

Transmission electron micrographs of apoptotic neutrophils ingested by macrophages

Human monocyte-derived macrophages were stimulated with CD44 antibody 5A4, washed, and then incubated with a suspension of aged neutrophils exactly as previously described. The cell monolayer was washed vigorously, macrophages were detached from the plastic wells by vigorous pipetting following 10 minutes' incubation at 37°C with trypsin-EDTA (Gibco), washed twice, fixed in freshly prepared 3% glutaraldehyde in 0.1M sodium cacodylate buffer, and processed for transmission electron microscopy by Steve Mitchell at the Electron Microscopy Unit, Royal Dick Veterinary School, Edinburgh.

Cytocentrifuge preparations following macrophage phagocytosis of apoptotic neutrophils

Following the standard phagocytosis assay but prior to addition of glutaraldehyde, macrophages were detached from the plastic wells by vigorous pipetting following 10 minutes' incubation at 37°C with 200 µl per well trypsin-EDTA (Gibco). 150 µl of cell suspension from each wells was then cytocentrifuged onto separate glass slides, air dried, fixed in methanol, and stained with Diff-Quik, In parallel experiments phagocytosis of apoptotic neutrophils by human macrophages was assessed counting both the percent phagocytosis "in-well" and of cytocentrifuge preparations.

Labelling of bound but non-ingested neutrophils with CD15 antibody

Human monocyte-derived macrophages in 48 well plates were stimulated with CD44 antibody 5A4 and then incubated with a suspension of aged neutrophils before washing, exactly as described above. The monolayers were fixed with 2% formaldehyde in PBS, washed, and blocked with neat rabbit serum or human AB serum for 10 minutes. Cells were then incubated at room temperature for 30 minutes each with 1:100 MC2 (IgM mouse anti-CD15), 1:50 biotinylated goat anti-mouse IgM (Amersham), and 1:50 streptavidin-FITC (Dako), with three washes with PBS between each step. Phagocytosis of apoptotic neutrophils was assessed using the standard "in-well" counting method, with or without adjustment for bound but non-ingested cells that were visualised on the same inverted microscope fitted with a fluorescent lamp.

Cytokine release assay

Four to six day old human monocyte-derived macrophages in 48 well plates were pre-incubated for four hours in medium alone (control) or medium containing 50 ng/ml E. coli O18 lipopolysaccharide. Cells were then washed three times prior to addition of CD44 antibody 5A4 (1:5 supernatant) or medium alone for 30 minutes. After two further washes, macrophages were incubated at 37°C for 60 minutes with 0.5 ml Iscove's medium alone or 0.5 ml Iscove's medium containing (a) $5x10^6$/ml aged neutrophils; (b) $5x10^6$/ml of human peripheral blood erythrocytes that had been opsonized with IgG by incubation for 60 minutes at 37°C in cation-free PBS containing 1:1000 rabbit polyclonal anti-human erythrocyte membrane antibody (Dako); or (c) 1 mg/ml zymosan A (Sigma) that had been washed, solicited for a few seconds, and opsonized by incubation for 60 minutes at 37C in cation-free PBS containing 1:2 pooled human AB serum. Non-ingested particles were washed away, and 0.5 ml of fresh serum-free Iscove's medium was added. The medium was harvested at three hours, replaced with fresh medium, and then harvested again at 18 hours. Parallel wells were processed for assessment of phagocytosis. Supernatants were centrifuged at 220g to remove cellular debris and stored at –80°C prior to cytokine ELISA.

TNF-α ELISA

TNF-α ELISA was performed using commercially available reagents (R&D systems, U.K.) according to the following protocol:

Coat: 100 µl/well 1:20 anti-TNF-α in 50 mM $Na_2CO_3/NaHCO_3$ pH 9.6 for 2h at 35C. Remove.
Block: 200 µl/well blocking buffer (1% BSA in coating buffer) for 15 mins at room temperature
Wash: 3x wash in 0.1% Tween 20 in TBS pH 7.4

Samples:100 µl/well hr TNF-α 3.12-400 pg/ml or samples in 1% BSA/ TBS pH 7.4 for 16h at 4C or 2h at room temperature (with shaking)
Wash x3
2nd Ab: 100 µl/well 1:20 anti-TNF-α-peroxidase in 1% BSA/ 10mM EDTA/ TBS pH 7.4 for 4h (2h with shaking) at room temperature
Wash x3
Develop:100 µl/well 0.1 mg/ml TMB in 100 mM sodium acetate/citrate pH 4.9 for 1h at room temperature
Stop: 100 µl/well 1M H_2SO_4
Measure :absorbance at 450 nM

IL-8 and IL-10 ELISA

Measurement of IL-8 and IL-10 concentrations in cell culture supernatants was performed according to the manufacturer's instructions using commercially available self contained kits (R&D systems, U.K.). Both methods used a sandwich ELISA technique with an immobilised unlabelled antibody, a range of protein standard concentrations, and a soluble peroxidase-labelled antibody.

Preparation of macrophage lysates

Human monocyte derived macrophages were grown adherent to six well plates (Costar) in a similar method to that described for 48 well plates. Approximately 10^6 human monocytes in a suspension of freshly isolated peripheral blood mononuclear cells were added to each well of a six well plate, allowed to adhere for 1 hour at 37°C, washed to remove contaminating lymphocytes, and cultured for six days in Iscove's medium containing 10% autologous serum with a change of medium on the third day. The mature macrophages were then washed, incubated with CD44 antibody, control antibody, or PMA in Iscove's medium for the required time, and then washed again. For some experiments, macrophages were lysed with 150 µl/well of 4xLaemmli SDS sample buffer for five minutes. The lysates were then aspirated and stored at –80°C prior to use. For immunoprecipitation experiments, macrophages were lysed with 200 µl/well 150 mM NaCl/25 mM Tris pH 7.6 containing 1% Nonidet P-40 (NP-40), 0.5% deoxycholate, protease inhibitor cocktail (Boehringer), and phosphatase inhibitors (1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 50mM sodium fluoride, and 10mM β-glycerophosphate). The lysates were incubated on ice for 15 minutes, the macrophage monolayer was then scraped with a plastic scraper, and the lysates aspirated into plastic tubes for a further 15 minutes on ice. The samples were cleared of detergent-insoluble material by centrifugation at 10^5g for 15 minutes at 4°C, and the detergent-soluble supernatants were stored at –80°C prior to use.

Dot blotting

200 µl samples containing proteins at 1.25 µg/ml or 1:80 dilutions of serum in PBS were applied to a nitrocellulose membrane using a dot blot manifold (for some experiments 3 µl dots of peptides at 1 mg/ml were applied with a pipette tip). The membrane was blocked for 2 hours in TBS/0.1% Tween 20 and then incubated with 1:100 Bob93 supernatant (serum free medium) for 30 minutes at room temperature with agitation. After washing the membrane was incubated with 1:4000 goat anti-mouse-HRP (Dako) and developed with ECL (Amersham).

Immunoprecipitation of paxillin and Western blotting

NP-40 soluble macrophage lysates (200 µl) were incubated with 1 µl paxillin (or ezrin, Lck, or phosphotyrosine) antibody (Affiniti; final concentration 1.25 µg/ml) for 1 hour on ice prior to addition of approximately 25 µl (pelleted volume) of washed anti-mouse Ig-agarose (Sigma) for 1 hour at 4°C with agitation. The pellets were washed three times with lysis buffer, boiled with 25 µl of 4x Laemmli reducing sample buffer, and 20 µl of the supernatant was run on a reducing 10% SDS-polyacrylamide gel. The proteins were blotted (0.5A for 1 hour) onto a nitrocellulose membrane which was then blocked for 2-24 hours in PBS containing 0.1% Tween 20 ± 1% BSA. The membrane was probed by incubation with 1:2500 (0.1 µg/ml) RC20 (anti-phosphotyrosine)-HRP (Affiniti) in PBS-0.1% Tween 20 for 1 hour. In some experiments, macrophage lysates were probed with anti-ezrin (1:250), anti-paxillin (1:10000), or anti-Lck (1:5000). Development was with enhanced chemiluminescence (Amersham). For some experiments blots were stripped with 0.1M glycine pH 2.9 for 20 minutes at room temperature followed by thorough washing with distilled water.

Protein biotinylation

Fetuin, asialofetuin, or human lactoferrin (Sigma) were dissolved at 2.5-3 mg/ml in PBS or deionised water and dialysed against 100 mM sodium bicarbonate pH 8.2 for 16 hours (3 changes) at 4°C. NHS-LC-Biotin (Pierce; 2-3 mg/ml in DMSO) was added to a total amount of 60 µg per mg protein, mixed well, and incubated for 3-4 hours at room temperature prior to extensive dialysis against PBS. Experiments using 120 µg of biotin per mg protein resulted in a conjugate that demonstrated relatively high non-specific binding in flow cytometry.

Labelling of fetuin with FITC

10 mg of bovine fetuin (Sigma F3004) was dissolved in 3mls deionised water and then dialysed against 100 mM sodium bicarbonate pH 8.25 for 16 hours (3 changes) at 4°C. Fluoroscein isothiocyanate (FITC; Sigma) was dissolved at 1.5 mg/ml in DMSO and added dropwise to a total volume of 45 µl per ml of protein solution. The mixture was then incubated for 2 hours at room temperature in the dark. The FITC-protein solution was slowly added to a G25 sepharose (Pharmacia) column that had been equilibrated with PBS. Unconjugated FITC was retained in the column and the eluent containing FITC-fetuin was collected in fractions. The protein concentration in each fraction was determined using a Lowry protein assay (Pierce) according to the manufacturer's instructions. 10 µl standards (0.1-0.5 mg) and 10 µl samples (diluted 1:5 in PBS) were added to 200 µl reagent (1 part reagent B plus 49 parts reagent A) in wells of a 96 well plate and incubated for 30 minutes at 37°C before measuring absorbance. Protein concentrations in seven serial fractions are shown in figure 1.

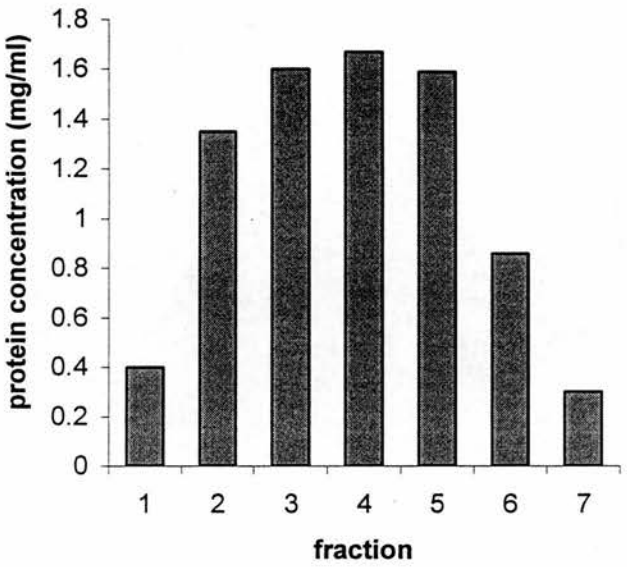


FIGURE 1
Purification of FITC fetuin on a G25 column
Seven serial fractions of eluent were subjected to protein assay using a Pierce Lowry kit.

Affinity isolation of fetuin-binding proteins

Cells were washed three times with PBS and surface proteins were labelled by incubation with 0.1 mg/ml NHS-LC-biotin (Pierce) for 1 hour at room temperature according to the manufacturer's instructions. The cells were washed twice with PBS and lysed on ice for 30 minutes at a concentration of 10^7/ml in Tris-buffered saline pH 7.5 containing 1%NP-40 and double strength protease inhibitor cocktail (Boehringer). The detergent-insoluble material was removed by centrifugation at 10^5g for 20 minutes and the lysates were stored at –80°C prior to use. Approximately 25 µl of washed fetuin-agarose (Sigma) was incubated with 200 µl of lysate for 2-4 hours at 4°C with agitation. The pellets were washed three times with lysis buffer, boiled with 25 µl of 4x Laemmli reducing sample buffer, and 20 µl of the supernatant was run on a 10% SDS-polyacrylamide gel. The proteins were blotted (0.5A for 1 hour) onto a nitrocellulose membrane which was then blocked for 2-24 hours in PBS containing 0.1% Tween 20. The membrane was probed by incubation with 1:5000 strepavidin-HRP (Dako) in PBS-0.1% Tween 20 for 1 hour and developed using enhanced chemiluminescence (Amersham).

Lectin binding

Labelled lectins were from Sigma, except for SNA-FITC and MAL2-biotin from Vector Laboratories, Peterborough, U.K..

Aged human peripheral blood neutrophils (approximately 50% apoptotic) were incubated for 30 minutes on ice with neat 3G8 (anti-CD16) supernatant, washed, and then incubated for 30 minutes with 1:40 $F(ab')_2$ goat anti-mouse PE together with the FITC-labelled lectin (except for MAL2 where the biotinylated lectin was used followed by 1:50 streptavidin-FITC for 30 minutes). The lectins used were concanavalin A (ConA from Concanavia ensiformis; principal ligand mannose); wheat germ agglutinin (WGA from Triticum vulgaris; $(N\text{-acetylglucosamine})_2$); peanut agglutinin (PNA from Arachis hypogaea; galactose); Lotus lectin (LTL from Tetragonolobus purpureas; fucose); Helix pomatia (HPA; N-acetylgalactosamine); Limulus polyphemus (LPA; sialic acid); elderberry lectin (SNA from Sambrucus nigra; α2-6 sialic acid); and Maackia amurensis (MAL2; α2-3 sialic acid). The cells were washed and dual colour flow cytometric analysis was performed following appropriate colour compensation. An effect of bound 3G8 antibody on subsequent lectin binding was excluded by examining lectin binding to a mixed population of 3G8-labelled and unlabelled freshly isolated neutrophils.

Statistical analysis

Results are presented as mean ± SEM, and n=number of independent experiments using cells from different donors. Differences were analysed by ANOVA and the Tukey multiple comparison test using Graphpad Instat® software (San Diego, CA).

CHAPTER 3
THE ROLE OF MACROPHAGE CD44 IN PHAGOCYTOSIS OF APOPTOTIC CELLS

INTRODUCTION

Apoptosis of neutrophil granulocytes and their subsequent phagocytic clearance by macrophages are key processes in the successful resolution of inflammation. If the rate of apoptotic cell death in the inflamed tissue is so intense that phagocyte clearance capacity is exceeded, free apoptotic cells eventually undergo secondary necrosis, resulting in release of harmful cellular contents and damage to the surrounding tissue (Haslett et al., 1994). It has been hypothesised that inadequate clearance of apoptotic cells may contribute to the tissue damage and perpetuation of the inflammatory response that characterise a variety of chronic diseases. Recent experimental evidence from in vivo studies supports such a role for overwhelming apoptosis in such adverse tissue outcomes. For example, treatment of mice with intraperitoneal Fas antibody triggered a massive wave of apoptosis in the liver following ligation of Fas on the surface of liver cells, and the animals subsequently developed extensive hepatic necrosis and died (Ogasawara et al., 1993). Similarly, repeated induction of apoptosis in the rat lung culminated eventually in pulmonary scarring (Hagimoto et al., 1997), similar to that seen in human inflammatory lung diseases such as fibrosing alveolitis. The adverse outcomes in these experimental situations possibly arose because of failure of the tissue phagocytes to clear the load of apoptotic cells with which they were confronted. By identifying the physiological mechanisms that regulate phagocyte clearance capacity for apoptotic cells it may prove possible to repeat experiments such as these whilst concurrently augmenting phagocytic capacity in the hope of inducing a more favourable outcome. Ultimately, induction of granulocyte apoptosis combined with artificial enhancement of macrophage phagocytic capacity for apoptotic granulocytes may be a therapeutic possibility in human inflammatory diseases.

In order that the load of apoptotic neutrophils at an inflammatory site is matched by appropriate clearance activity, macrophage capacity for apoptotic cell phagocytosis is likely to be closely regulated. Macrophage phagocytic capacity may be influenced by soluble mediators such as cytokines (Ren et al., 1995), prostaglandins (Rossi et al., 1998), and steroid hormones (Liu et al., 1999). In addition, many aspects of cellular behaviour, including phagocytosis, are modulated by interactions of cells with the ECM via ligation of specialised adhesion receptors and subsequent transduction of intracellular signals (Brown, 1986; Giancotti, 1997). The composition of the ECM undergoes a series of changes in response to tissue injury (Gailit and Clark, 1994), which provides a mechanism for close regulation of function of influxing and resident inflammatory cells, including macrophages. Recently it has been discovered that adhesion of macrophages to the matrix component fibronectin significantly augmented their capacity for phagocytosis of apoptotic neutrophils, an effect that was only partially inhibited by antibodies to β1 integrins (McCutcheon et al., 1998). Other cellular receptors involved in adhesion to fibronectin include CD44 (Jalkanen and Jalkanen, 1992), a transmembrane glycoprotein of the cartilage link protein that is expressed by murine peritoneal macrophages (Camp et al., 1991; Culty et al., 1994), human monocytes and alveolar macrophages (Levesque and Haynes, 1996), and many other cell types (Lesley et al., 1993a). Furthermore, ligation of CD44 by alternative ligands such as hyaluronan or by monoclonal antibodies may

profoundly alter the behaviour of macrophages in the inflammatory response (Webb et al., 1990; Noble et al., 1993; Noble et al., 1996; McKee et al., 1996).

This chapter describes the characterisation of CD44 expressed by human monocyte-derived macrophages and the effect of CD44 ligation on macrophage phagocytosis of apoptotic cells.

RESULTS AND DISCUSSION

Expression of cell surface CD44

Expression of CD44 on the surface of human monocyte-derived macrophages was assessed by indirect immunofluorescence and flow cytometric analysis using monoclonal antibodies. Some antibodies recognise constant epitopes on the extracellular domain and bind to all isoforms of CD44, and these are subsequently referred to as pan-reactive CD44 antibodies (Lesley et al., 1993a; Dougherty et al., 1994). Other monoclonal antibodies bind specifically to the exon-specific membrane-proximal peptide sequences that are present in particular CD44 variant isoforms.

Human monocyte-derived macrophages demonstrated strong binding of the pan-reactive CD44 antibody 5A4, and also expressed significant levels of isoforms v3 and v10 (table I). CD44v7-8 was reproducibly undetectable on the macrophage surface despite apparent synthesis of the mRNA transcript by PCR analysis, suggesting that this isoform is either not translated or is present only intracellularly (table I). The presence of other CD44 variant isoforms was not specifically examined, although Levesque and Haynes have subsequently demonstrated the presence of CD44 v4, v6, and v9 isoforms on human inflammatory macrophages (Levesque and Haynes, 1996). The ability of cells to bind the matrix sugar hyaluronan is not directly associated with CD44 expression and seems to depend on the cell type, degree of cell activation, and amount of glycosylation of CD44 (Lesley et al., 1993a). The monoclonal antibody 7F4 is unusual in that it seems to recognise an epitope on CD44 that is a marker for hyaluronan binding, although it does not bind itself to the hyaluronan-binding site and does not inhibit hyaluronan binding (Dougherty et al., 1994). Antibody 7F4 bound reasonably strongly to human monocyte-derived macrophages (Table I), which is consistent with the reported capability of human macrophages to bind labelled hyaluronan (Culty et al., 1994).

Monoclonal antibody	Macrophage antigen	Antigen expression (relative mean fluorescence)
MOPC31C	None	1.8 ± 0.1
5A4	all CD44 isoforms	307.0 ± 77.2
7F4	CD44 hyaluronan binding-associated epitope	119.0 ± 59.2
3G5	CD44v3	7.3 ± 1.2
VFF-17	CD44v7-8	2.6 ± 0.3
2G1	CD44v10	37.1 ± 3.6

TABLE I
Macrophage expression of CD44
Expression of macrophage CD44 isoforms was determined by analysis of antibody binding using indirect immunofluorescence and flow cytometry. Results are expressed as mean ± SEM of 3-6 independent experiments.

Immunoprecipitation of macrophage CD44 with monoclonal antibody 5A4

To further characterise macrophage CD44 and the CD44 antibodies that were to be used subsequently in functional studies, CD44 was immunoprecipitated from surface-labelled macrophage lysates. The pan-reactive CD44 monoclonal antibody 5A4 immunoprecipitated a surface protein of apparent molecular weight 95kDa (figure 1) consistent with CD44S (Camp et al., 1991; Lesley et al., 1993a). Additional higher molecular weight bands were especially prominent in some experiments and probably represented alternatively spliced isoforms (Figure 1).

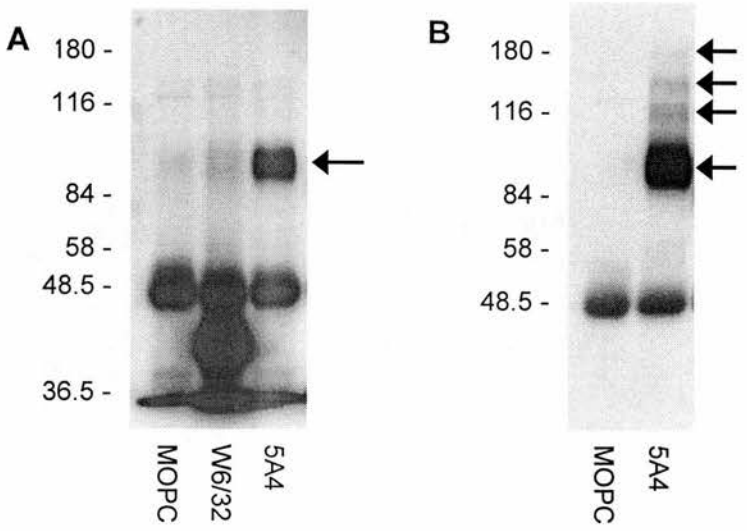


FIGURE 1

Immunoprecipitation of CD44 with monoclonal antibody 5A4

(A) Human monocyte-derived macrophages were surface labelled with NHS-LC-Biotin and then lysed with Tris-buffered saline (pH 7.5) containing 1% NP-40 and protease inhibitors. The lysates were incubated with monoclonal antibodies MOPC (non-binding control), W6/32 (MHC class I; control), or 5A4 (CD44) followed by anti-mouse Ig-agarose. The pellets were washed five times with lysis buffer and then boiled in reducing Laemmli SDS sample buffer. Immunoprecipitates were run on a 10% polyacrylamide gel and electroblotted onto nitrocellulose. The products were visualised by incubation with streptavidin-HRP and development using enhanced chemiluminescence. CD44 was immunoprecipitated by 5A4 as a band of apparent molecular weight 95 kD (arrow). W6/32 immunoprecipitated a protein of apparent molecular weight 40 kD, consistent with MHC class I. Antibody heavy and light chain bands are present in all three lanes.

(B) Macrophage lysates from a different donor were subjected to immunoprecipitation as in (A). Here a number of higher molecular weight bands are identified along with "standard" CD44 (arrows), probably representing alternatively spliced isoforms.

Role of macrophage CD44 in the phagocytosis of apoptotic neutrophils

It is hypothesised that temporal alterations in the composition of the ECM following tissue damage lead to differential ligation of macrophage adhesion receptors that then modulates their capacity for apoptotic cell phagocytosis (Brown, 1986; Gailit and Clark, 1994). CD44 has been reported to bind a variety of ligands in the ECM in addition to certain cell surface proteins and soluble extracellular proteins (Lesley et al., 1993a; Toyama-Sorimachi et al., 1995; Weber et al., 1996; Toyama-Sorimachi et al., 1997), and functional consequences of CD44-ECM interactions may be mediated by intracellular signalling pathways (Rothman et al., 1993; Galandrini et al., 1996; Taher et al., 1996). Binding of a ligand to its cell surface receptor may be mimicked by binding of an antibody, and previous studies of CD44 ligation have used CD44 monoclonal antibodies in this way (Webb et al., 1990; Rothman et al., 1993). To assess the role of macrophage CD44 in the phagocytosis of apoptotic neutrophils in vitro, human monocyte-derived macrophages were pre-incubated with CD44 monoclonal antibody for 20 minutes immediately prior to assessment of phagocytosis of apoptotic neutrophils using a well characterised phagocytic assay (Newman et al., 1982; Savill et al., 1989). A control antibody (W6/32, against a non-polymorphic determinant on MHC class I) was chosen that, like 5A4, bound strongly to human monocyte-derived macrophages.

Binding of CD44 antibody 5A4 resulted in a dramatic increase in macrophage phagocytosis of apoptotic neutrophils (figure 2), whereas control antibody W6/32 had no significant effect.

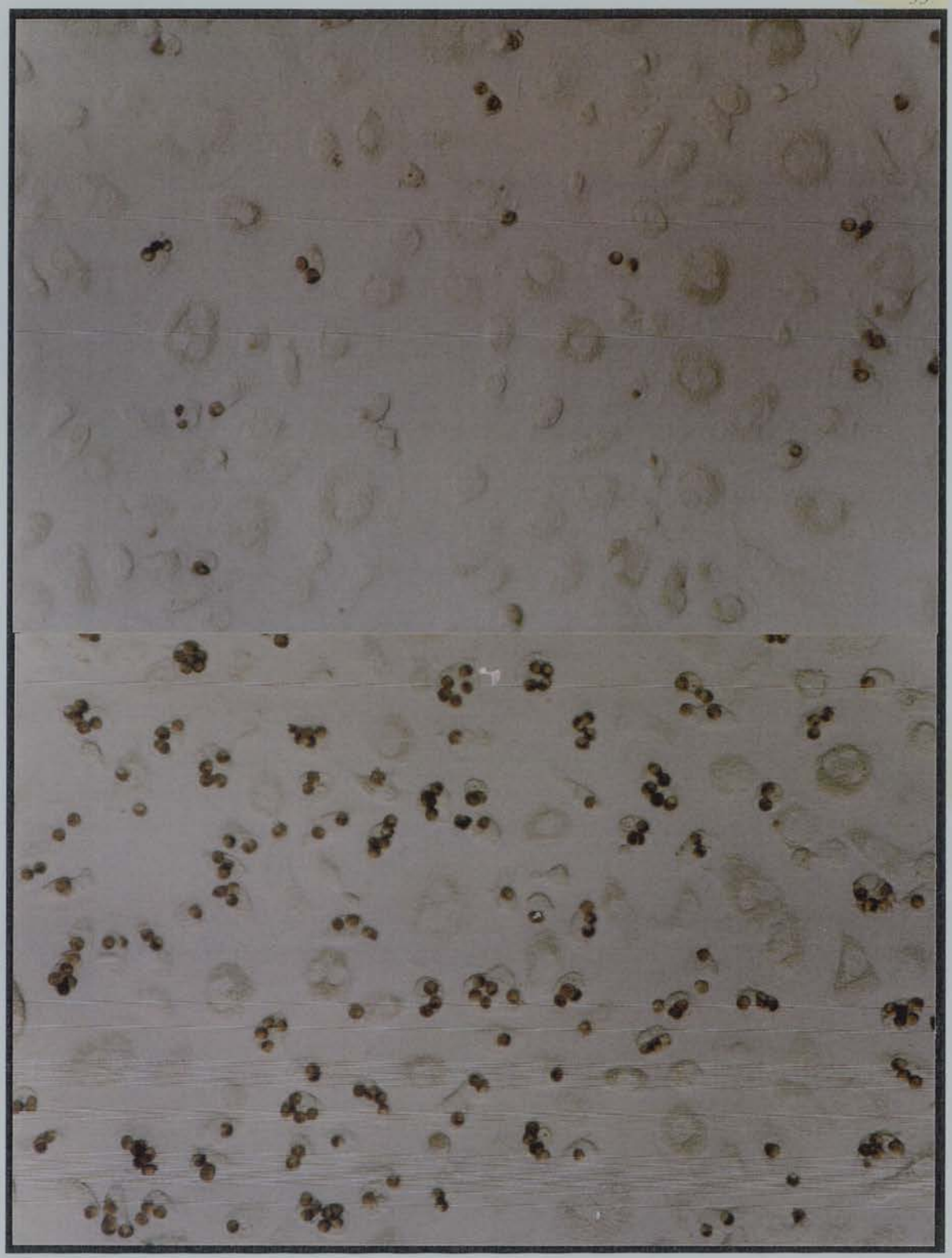


FIGURE 2

CD44 antibody 5A4 augments macrophage phagocytosis of apoptotic neutrophils.

Representative micrographs from one experiment showing macrophage phagocytosis of apoptotic neutrophils following incubation with medium alone (upper panel) and CD44 antibody 5A4 (lower panel). After thorough washing and fixation cells were stained for myeloperoxidase. Apoptotic neutrophils were 100% myeloperoxidase-positive (brown reaction product), while macrophages were myeloperoxidase-negative. 5A4-treated wells show an increase both in the proportion of macrophages that have phagocytosed apoptotic neutrophils and in the number of neutrophils per phagocytosing macrophage. In this experiment, 16.0% of control macrophages and 44.7% of 5A4-treated macrophages phagocytosed apoptotic neutrophils.

Measurement of the percentage of macrophages that ingest one or more particles has become the traditional method by which phagocytosis of apoptotic cells is assessed (Savill et al., 1989). However, because the control rate of phagocytosis exhibits wide variation between different macrophage donors (range 4.4 to 66.7% in my series of experiments), phagocytosis has been expressed as a percentage of control for each experiment in order that valid comparisons can be made between different experiments (Savill et al., 1989; Savill et al., 1993). Binding of CD44 antibody 5A4 resulted in a 2.3-fold increase in the proportion of macrophages that phagocytosed one or more apoptotic neutrophils (figure 3). Pre-incubation of macrophages with saturating concentrations of other pan-reactive CD44 antibodies (3C9, 3C12, and F10.44.2) resulted in a similar increase in the proportion of macrophages that phagocytosed apoptotic neutrophils (Hart et al., 1997). In contrast, monoclonal antibodies specific for integrin α_5, β_1, or β_2 subunits had no demonstrable effect (I. Dransfield, unpublished observations).

The analysis was extended to include other antibodies that recognise specific variant isoforms of CD44 expressed by human monocyte-derived macrophages. Interestingly, CD44 antibodies that define CD44v3 (antibody 3G5) or CD44v10 (2G1) isoforms, or a hyaluronan binding-associated epitope (7F4), failed to augment phagocytosis (figure 3), despite binding to macrophages (table I). However, it is difficult to completely discount a role for specific isoforms of CD44 in regulation of phagocytosis of apoptotic neutrophils based on the lack of effect of single antibodies, and further studies using a more extensive panel of CD44 antibodies would be required. Interestingly, there was no effect of antibody 61D3 that recognises CD14 and which has been reported to inhibit recognition of apoptotic lymphocytes by human macrophages (Flora and Gregory, 1994; Devitt et al., 1998).

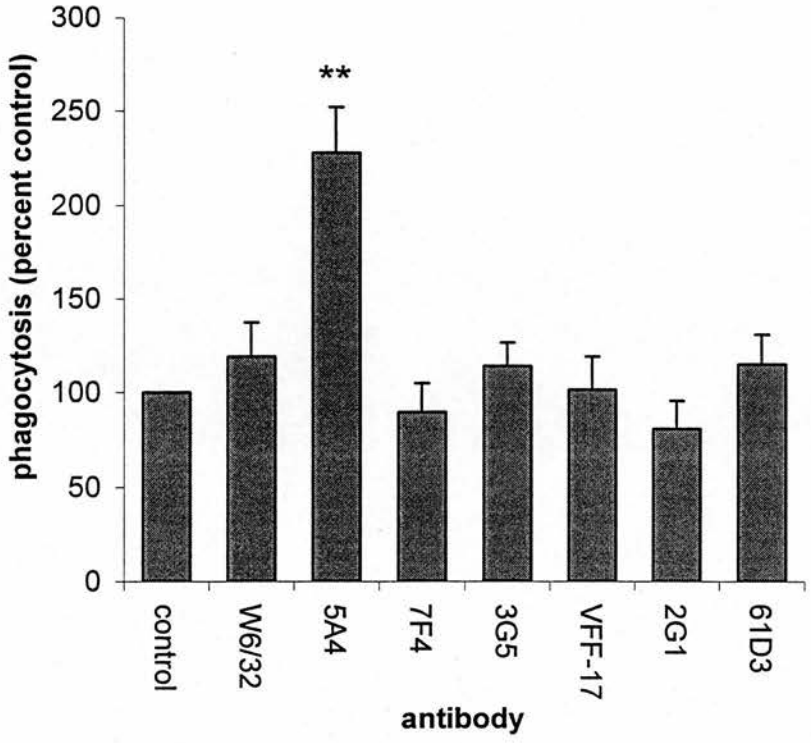


FIGURE 3.
The effect of CD44 antibodies on macrophage phagocytosis of apoptotic neutrophils.
Macrophages were incubated with the indicated antibody for 20 minutes, washed, and incubated with a suspension of aged neutrophils for 30 minutes. After vigorous washing, the percentage of macrophages that had phagocytosed one or more apoptotic neutrophils was enumerated using a light microscope. In this series of experiments 19.9±1.6% of control macrophages phagocytosed apoptotic neutrophils. **P<0.01 compared with control (n=4-28).

It could be argued that although ligation of macrophage CD44 with pan-reactive CD44 antibodies such as 5A4 dramatically increased the proportion of macrophages that ingested apoptotic neutrophils, the total number of phagocytosed particles could be unchanged or even diminished if the average number per phagocyte was lower than that under control conditions. To exclude this possibility, in a small series of experiments the total number of ingested apoptotic neutrophils, expressed per 100 macrophages as the "phagocytic index" (Fadok et al., 1992b), was also measured. Table II demonstrates that treatment with CD44 antibody 5A4 also increased macrophage phagocytic capacity as assessed by the phagocytic index.

	(A) percent phagocytosis	(B) phagocytic index	(C) average per MP
control	26.9 ± 7.5	57.7 ± 24.8	2.0 ± 0.4
5A4	47.9 ± 5.3	133.0 ± 34.6 *	2.7 ± 0.6

TABLE II

CD44 antibody increases the total ingested load of apoptotic neutrophils

Human monocyte-derived macrophages were incubated with CD44 antibody 5A4 for 20 minutes prior to assessment of phagocytosis of apoptotic neutrophils as previously described. Three parameters of phagocytic activity were assessed: (A) the percentage of macrophages which ingested one or more apoptotic neutrophils; (B) the total number of neutrophils ingested per 100 macrophages (phagocytic index); (C) the average number of neutrophils ingested per "phagocytic" macrophage (MP). Results are presented as mean ± SEM of three independent experiments. * $P<0.05$.

Augmentation of phagocytosis across a range of basal phagocytosis rates

Human monocyte-derived macrophages exhibit a large variation in the basal phagocytic capacity, which seems to depends on a number of factors including the macrophage donor, in vitro culture conditions, and duration of culture, but interestingly appears to be independent of the proportion of apoptotic cells within the aged neutrophil population that is fed to the macrophages in the phagocytosis assay (figure 4). Although the number of experiments may have been too small to observe a small but significant association between the proportion of apoptotic cells and phagocytosis rates, these observations conflict with previously published studies in which a clear correlation was reported (Stern et al., 1992). Asynchronous apoptosis within a population of ageing neutrophils means that at the time of cell harvesting (20 hours of in vitro culture) there will be cells at all stages of apoptosis, both early and late. The physical and chemical characteristics of early and late apoptotic cells may be very different, as may the mechanisms employed for phagocyte recognition. If, for example, early apoptotic cells were engulfed by macrophages more avidly than cells that had proceeded to late apoptosis then, depending on the time of harvesting of the cells, the correlation between apoptosis and ingestion may be positive, negative, or absent.

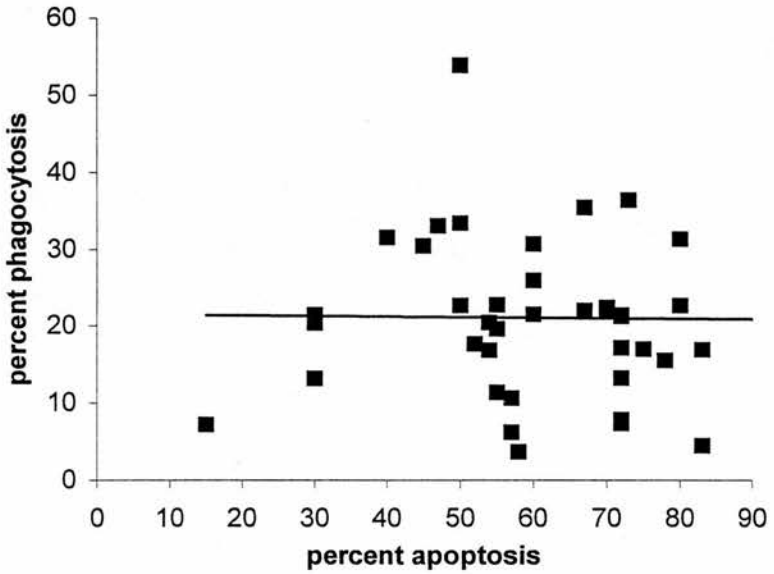


FIGURE 4
Lack of correlation between percent apoptosis and percent phagocytosis
The proportion of apoptotic cells in the suspension of aged neutrophils fed to macrophages was assessed by binding of CD16 antibody or annexin V in flow cytometry. The percentage of apoptotic cells was plotted against the percent phagocytosis by control (untreated) macrophages for a series of 43 experiments (line of best fit is shown; R^2=0.0002).

To determine whether macrophage CD44 ligation increased phagocytosis of apoptotic neutrophils across a range of baseline phagocytic activity, the control rate of phagocytosis was plotted against 5A4-augmented phagocytosis for a series of 72 experiments (figure 5). It can be seen that 5A4-augmented phagocytosis was approximately linearly correlated with basal phagocytosis up to a basal percentage phagocytosis of at least 40-50%. Only in the relatively few experiments with higher baseline rates did CD44 ligation have a diminishing, although still significant, stimulatory effect.

The data presented here clearly show that CD44 ligation on human macrophages by pan-reactive antibodies rapidly and vastly augments phagocytosis of apoptotic neutrophils in vitro, and suggest a key role for CD44 in regulation of phagocytosis of apoptotic neutrophils in vivo. These data provide the first evidence that macrophage phagocytosis of apoptotic neutrophils can be rapidly (within minutes) augmented following ligation of specific macrophage surface receptors, and raise the possibility that distinct CD44 isoforms may exert differential effects.

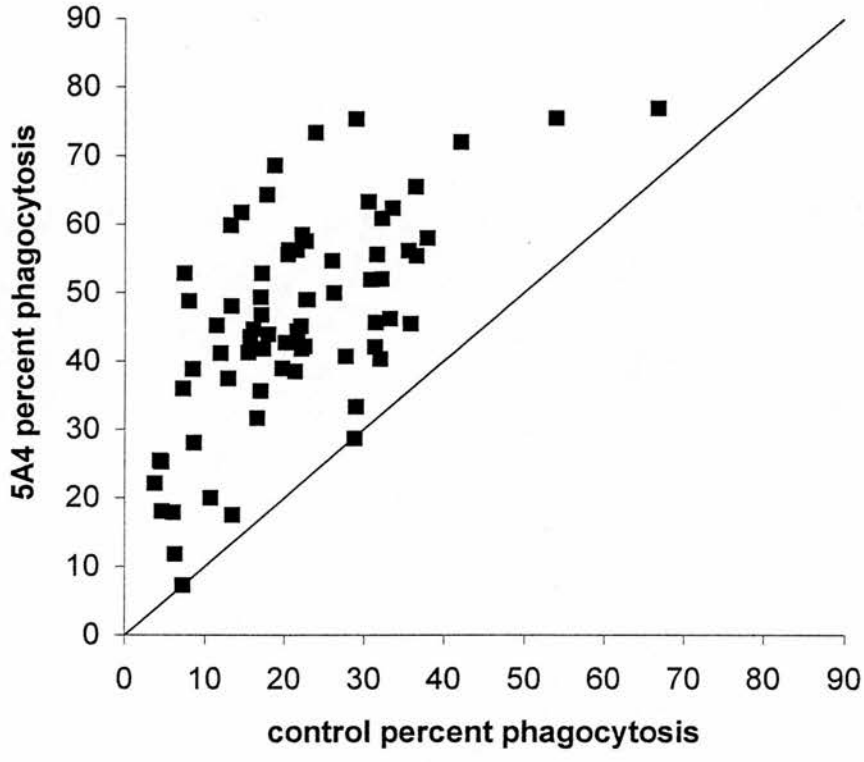


FIGURE 5
Relationship of 5A4-augmented phagocytosis to baseline (control) phagocytosis
5A4-augmented phagocytosis (percentage of macrophages ingesting one or more apoptotic neutrophils) was plotted against control phagocytosis (by untreated macrophages) for a series of 72 separate experiments (line plotted is x=y).

Discrimination of bound from ingested particles

The process of phagocytosis of any particle comprises an initial binding event, traditionally believed to involve specific receptors on the phagocyte surface, followed by a complex series of cytoskeletal reorganisations and plasma membrane extensions that lead to particle engulfment (Howard and Watts, 1994; Brown, 1995; Allen and Aderem, 1996a). Little attention has been focussed on discrimination between the processes of binding and ingestion in the published studies that have used in vitro assays of apoptotic cell phagocytosis. In my series of experiments the macrophage monolayer was washed very vigorously at the end of the assay to remove any non-ingested neutrophils. Indeed, when viewing the completed interaction with the microscope, there was typically no evidence of "rosetting" that classically indicates binding of particles to the phagocyte surface. However, it remained possible that the apoptotic cells apparently ingested following CD44 ligation were in fact simply bound to the free surface of the macrophages, even though they were completely within the outline of the macrophage plasma membrane in the majority of cases. Three different approaches were taken to determine whether the apoptotic neutrophils were indeed truly ingested or simply bound to the macrophage surface following CD44 ligation: transmission electron microscopy, cytocentrifugation and microscopy of detached macrophages following the phagocytosis assay, and selective labelling of non-ingested particles.

Transmission electron microscopy

When cells were processed and examined by transmission electron microscopy following the phagocytic interaction apoptotic neutrophils could be clearly visualised within macrophage phagocytic vacuoles (figure 6). Although this observation suggested that true phagocytosis had occurred, quantitation of phagocytosis was not feasible using this method.

Cytocentrifuge preparations

As a second approach, macrophages were detached from the wells following the phagocytic interaction (but prior to fixation), and cytocentrifuged onto glass slides. The cells were then fixed with methanol and stained with Diff-Quik with or without DMB/H_2O_2 to stain neutrophil myeloperoxidase (figure 7). Using this method, ingested apoptotic neutrophils could be clearly discriminated from bound cells and the proportion of macrophages that had ingested apoptotic neutrophils could be quantitated and compared with results obtained using the standard in-well counting technique in parallel experiments. The results are presented in table III. The percentage phagocytosis results derived from counting cytospins were lower than those from in-well counting, indicating that some binding without ingestion had occurred in both control and CD44 antibody-treated wells. However, the proportional increase in phagocytosis following CD44 ligation was similar using either method. These results confirm that CD44 ligation leads to a true increase in phagocytosis of apoptotic neutrophils.

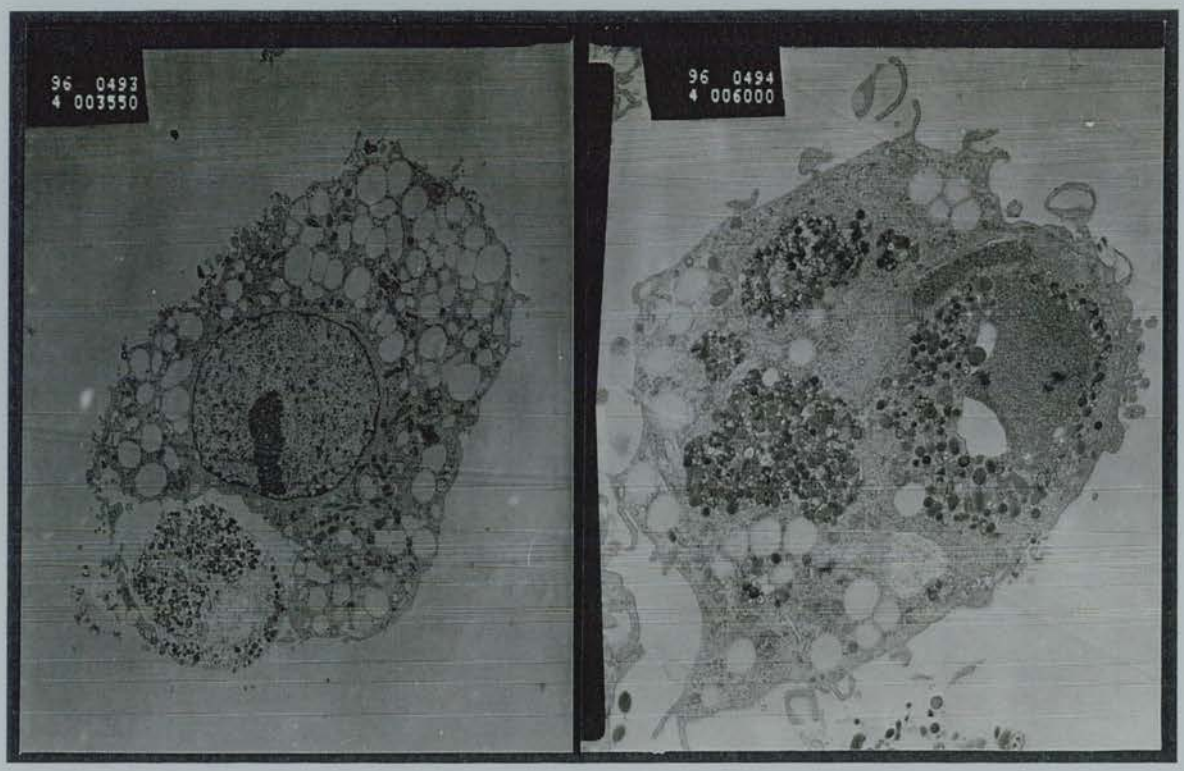


FIGURE 6

Transmission electron micrographs of apoptotic neutrophils ingested by macrophages following stimulation with CD44 antibody

Human monocyte-derived macrophages were stimulated with CD44 antibody 5A4, washed, and then incubated with a suspension of aged neutrophils as previously described. The cell monolayer was washed vigorously, macrophages were detached from the plastic wells by vigorous pipetting following incubation with trypsin-EDTA, washed, fixed with 3% glutaraldehyde in 0.1M sodium cacodylate buffer, and processed for transmission electron microscopy.

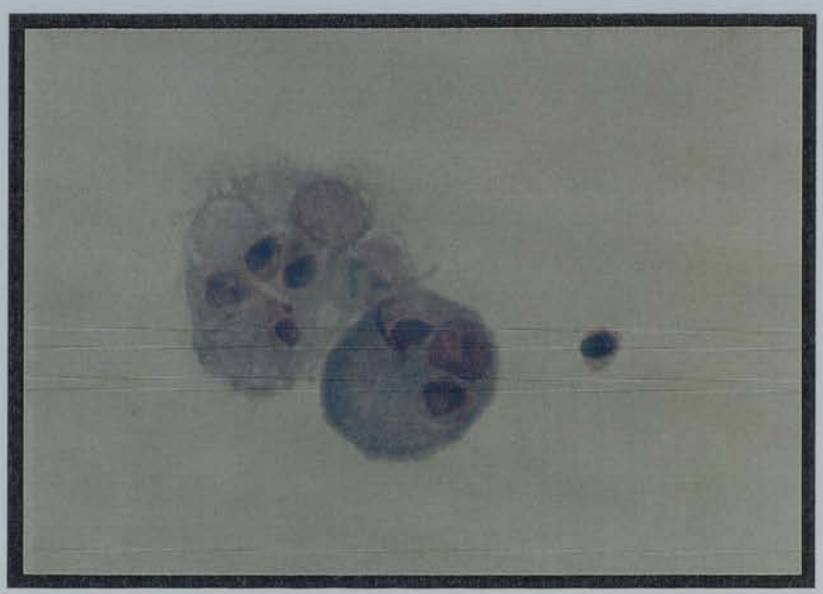


FIGURE 7

Stained cytocentrifuge preparation of ingested apoptotic neutrophils within human macrophages

Human monocyte-derived macrophages were incubated with aged neutrophils as described, washed vigorously, detached from the plastic plates with trypsin-EDTA, cytocentrifuged onto glass slides, and stained.

	In-well	Cytospin
Control	27.7 ± 3.6	23.2 ± 7.6
5A4	59.3 ± 14.2	46.6 ± 9.5
increment (5A4-control)	31.6 ± 10.5	23.4 ± 2.1
percent increase (5A4/control*100)	215 ± 19.1	221 ± 34.7

TABLE III

Comparison of measurements of macrophage phagocytosis of apoptotic neutrophils using the standard "in-well" and cytospin counting methods.

In parallel experiments phagocytosis of apoptotic neutrophils by human macrophages was assessed using either the standard "in-well" counting method, or macrophages were detached, cytocentrifuged onto glass slides, stained, and then the percent phagocytosis was counted. Results are presented as mean percent phagocytosis ± SEM (n=3).

Selective staining of non-ingested neutrophils

As a third approach, bound but non-ingested neutrophils were selectively stained "in-well". Cells were fixed in formaldehyde following the standard phagocytosis assay, and then bound but non-ingested neutrophils were selectively stained by indirect immunofluorescence with CD15 antibody MC2 (figure 8). CD15 is a cell surface carbohydrate that is expressed by neutrophils but not by macrophages. Since the cells had been fixed with formaldehyde and had therefore not been artificially permeabilised, the IgM antibody MC2 bound to CD15 present only on non-ingested neutrophils, but did not have access to neutrophils that were completely within macrophage phagocytic vacuoles. Using this method it was possible to adjust the standard in-well counts to take account of the number of bound but non-ingested neutrophils. As is shown in table IV, this adjustment had essentially no effect on the proportion of macrophages enumerated as having ingested apoptotic cells, although the average number of apoptotic particles per macrophage was reduced. These observations are consistent with the proposal that within the phagocytosis assay there are always some apoptotic cells that have bound to macrophages but have not yet been internalised, and that these cells were almost always associated with macrophages that had already ingested other apoptotic cells.

		PERCENT PHAGOCYTOSIS		PHAGOCYTIC INDEX	
		Standard	**Adjusted**	**Standard**	**Adjusted**
Expt 1	**Control**	41.9	41.9	107	107
	5A4	50.1	49.3	197	191
Expt 2	**Control**	18.4	18.1	37	37
	5A4	37.9	37.5	78	76

TABLE IV

Measurements of macrophage phagocytosis of apoptotic neutrophils before and after adjustment for the number of bound neutrophils assessed by binding of CD15 antibody.

Human monocyte-derived macrophages were stimulated with CD44 antibody 5A4 and then incubated with a suspension of aged neutrophils before washing, fixation with 2% formaldehyde, and blocking of Fc receptors with rabbit serum. Bound but non-ingested neutrophils were selectively labelled by incubation with CD15 monoclonal antibody MC2, and visualised using indirect immunofluorescence with a FITC label. Phagocytosis of apoptotic neutrophils was assessed using the standard "in-well" counting method, with or without adjustment for bound but non-ingested cells that were visualised on the same inverted microscope fitted with a fluorescent lamp. Results from two independent experiments are presented.

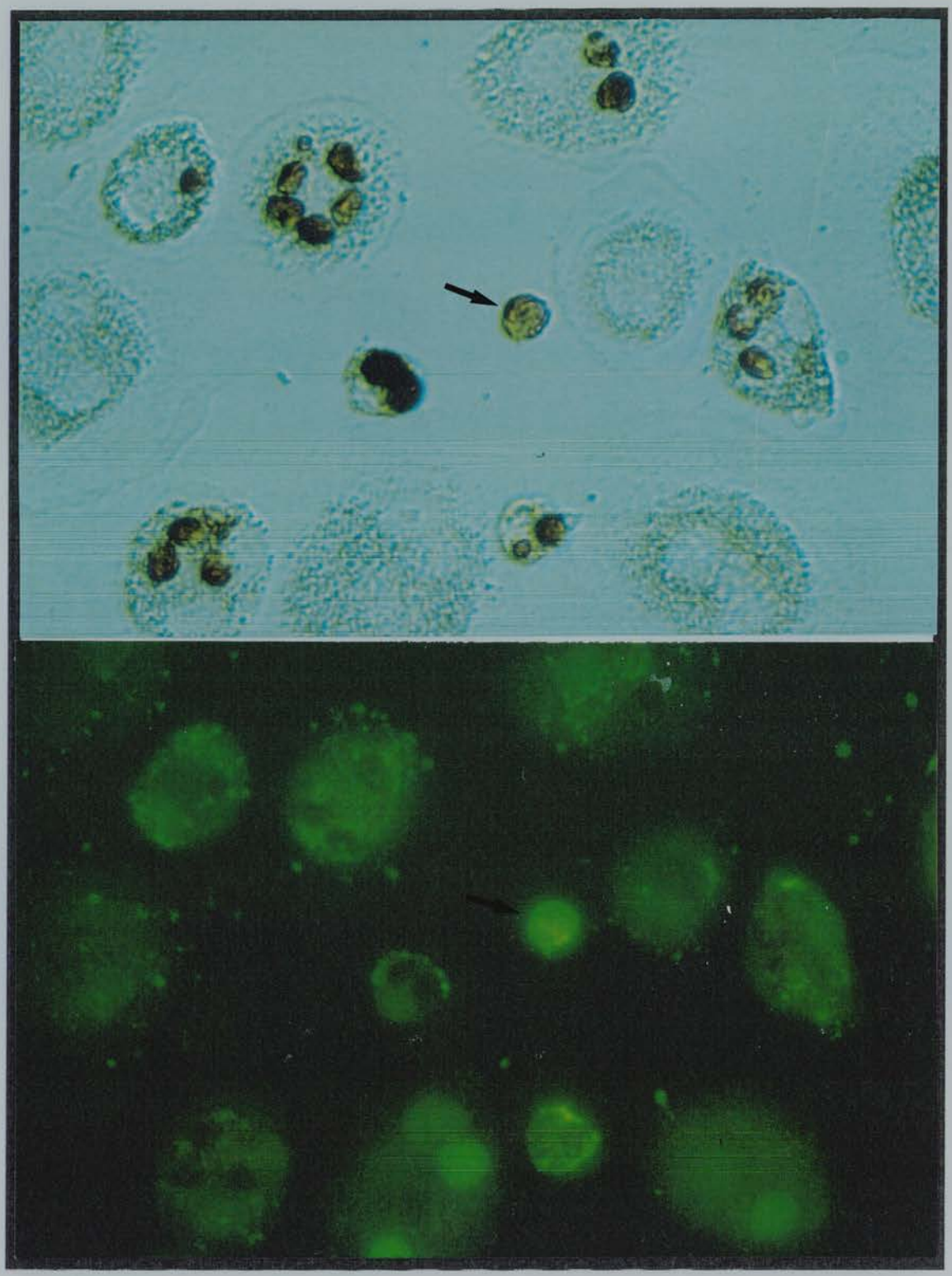


FIGURE 8

Selective staining of non-ingested neutrophils

Human monocyte-derived macrophages were stimulated with CD44 antibody 5A4 prior to addition of a suspension of aged neutrophils, and the standard phagocytosis assay was performed. The cell monolayer was washed and fixed with 2% formaldehyde, and Fc receptors were blocked with rabbit serum. Non-ingested neutrophils were selectively labelled by incubation with CD15 monoclonal antibody MC2, visualised using indirect immunofluorescence with anti-mouse IgM-FITC, and photographed on an inverted microscope. The same field has been photographed under standard light conditions (upper panel) and under fluorescent light with a FITC filter (lower panel). In the centre of the field is a non-ingested neutrophil. There is some background staining of the macrophages, but no staining of ingested neutrophils.

There is discrepancy between the phagocytosis rate assessed by labelling of non-ingested cells and that assessed on the cytocentrifuge preparations. This may be because it was not always easy to identify ingested apoptotic cells on the cytospins, and some non-ingested apoptotic neutrophils may not have been adequately visualised by CD15 labelling since neutrophil apoptosis is associated with down-regulation of CD15 (I. Dransfield, unpublished observations). Furthermore, "well fed" macrophages are fragile and may selectively undergo fragmentation during cytocentrifugation, leading to underestimation of the phagocytosis rate.

Augmented phagocytosis requires binding of bivalent CD44 antibody

To further address the mechanisms by which CD44 antibody acted to promote macrophage phagocytosis of apoptotic neutrophils the requirement for bivalent antibody binding was investigated. Monoclonal antibody 5A4 was purified and $F(ab')_2$ fragments were synthesised by pepsin digestion. Fab' fragments were then generated by reduction of the disulphide bonds with iodoacetamide (Dransfield et al., 1992). Purified Fab' and $F(ab')_2$ fragments were free of intact antibody as determined by gel electrophoresis and immunoblotting (figure 9), and bound to macrophages in a saturable manner in flow cytometric analysis.

When macrophages were pre-incubated with saturating concentrations of proteolytic fragments of CD44 antibody 5A4, augmented phagocytosis of apoptotic neutrophils was observed only with $F(ab')_2$ fragments, and not with Fab' fragments (figure 10). Since there was no detectable contamination of the $F(ab')_2$ preparation with intact antibody, these results discount a role for binding of CD44 antibody to macrophage Fc receptors in the augmentation of phagocytosis. A role for Fc receptors was further discounted by the lack of effect of a variety of other antibodies, including those of the mouse IgG2a isotype (such as W6/32) that exhibit highest affinity for human Fcγ receptors (Woof et al., 1986).

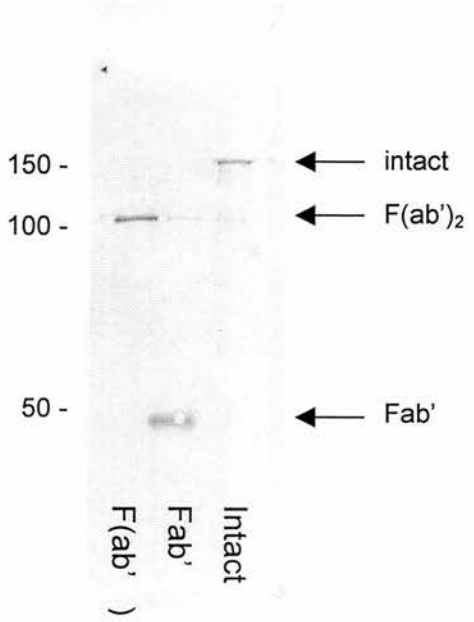


FIGURE 9
Western blot of Fab' and F(ab')$_2$ fragments of purified antibody 5A4
Monoclonal antibody 5A4 was purified from hybridoma supernatant on a protein A-agarose column. Purified antibody was subjected to pepsin digestion to prepare F(ab')$_2$ fragments, followed by iodoacetamide reduction to prepare Fab' fragments. Intact antibody and synthesised fragments were run on a non-reducing 10% polyacrylamide-SDS gel, blotted onto nitrocellulose, and visualised with anti-mouse Ig-HRP and 0.5 mg/ml diaminobenzidine/ 0.03% H_2O_2. MW markers (kDa) are presented on the left of the figure.

The lack of effect of Fab' fragments of CD44 antibody, despite their binding to human macrophages, indicates that bivalent antibody binding is required of the augmented phagocytosis of apoptotic neutrophils. This is an important observation because it alludes to the possible underlying mechanism of action of CD44 antibody in this situation. If simple masking of a CD44 epitope by intact antibody was responsible for the observed affect, then monovalent Fab' fragments would be expected to act similarly. Requirement for bivalent binding implies that receptor crosslinking is a prerequisite, which then leads to initiation of intracellular signal transduction (Sanchez-Mejorada and Rosales, 1998). In this series of experiments CD44 antibody was used as a convenient experimental substitute for the natural ligand(s) of CD44, and in a physiological situation it is likely that CD44 on the macrophage surface would be crosslinked by large multivalent ligands in the extracellular matrix. Moreover, the requirement for bivalent binding is similar to that reported for CD44 antibody-induced cellular adhesion (Lesley et al., 1993; Vermot-Desroches et al., 1995) and lymphocyte proliferation (Pierres et al., 1992). The mechanisms by which CD44 ligation promotes phagocytosis of apoptotic neutrophils are explored in the next chapter.

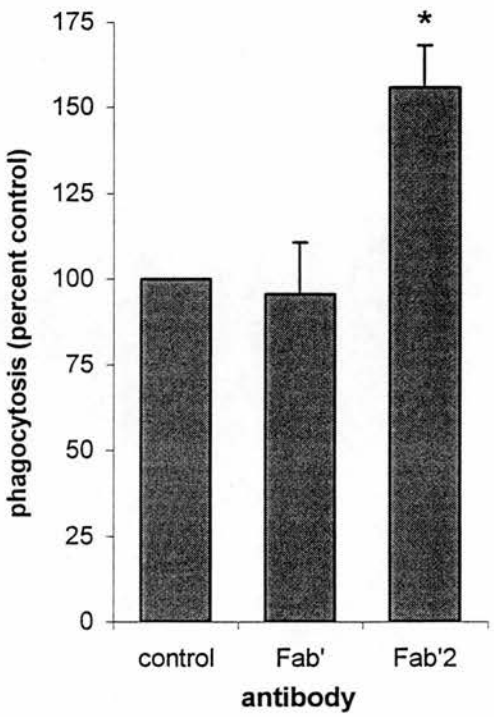


FIGURE 10
Macrophage phagocytosis of apoptotic neutrophils is augmented by F(ab')$_2$ but not Fab' fragments of CD44 antibody.
Macrophages were incubated with saturating concentrations of Fab' and F(ab')$_2$ fragments of CD44 antibody 5A4 prior to assessment of phagocytosis of apoptotic neutrophils. In this series of experiments control phagocytosis was 23.1±3.3%. *P<0.05 compared with control (n=4-10).

Specificity of augmented phagocytosis for apoptotic neutrophils

It is remarkable that phagocytosis of particles seems to be initiated following engagement of a huge variety of different receptors on the phagocyte surface. For example, in humans three different receptors for the Fc region of IgG may be involved in the ingestion of foreign particles that have been opsonized with antibody (Heijnen and van de Winkel, 1997). Similarly, coating of microbes with components of the complement cascade may lead to ligation of at least four distinct macrophage receptors (Ross, 1989). Phagocytosis of the opportunistic pathogen Pneumocystis carinii may be mediated by a mannose receptor (Ezekowitz et al., 1991), and blockade of either CD11b/CD18 ($\alpha_M\beta_2$; CR3), the mannose receptor, or a β-glucan receptor has been reported to inhibit uptake of yeast particles (Riches et al., 1988). Receptors involved in the uptake of "inert" particles such as latex beads or glutaraldehyde-fixed erythrocytes have been poorly characterised (Benoliel et al., 1980). Furthermore, at least seven different receptor classes have been reported to play a role in the recognition of apoptotic cells (see chapter 1).

Results presented in this chapter demonstrate that ligation of macrophage CD44 results in a rapid and profound increase in the phagocytosis of apoptotic neutrophils. Next it was determined whether CD44 antibody promoted macrophage phagocytosis of other particles. Erythrocytes coated with immunoglobulin G (EIgG) have been used as a control particle in a number of studies of apoptotic cell phagocytosis (Savill

et al., 1989a; Savill et al., 1993). However, in these studies greater than 90% of human macrophages phagocytosed EIgG (Savill et al., 1989a). In order to demonstrate a stimulatory effect on phagocytosis following CD44 ligation it was important to have lower basal levels of particle uptake, comparable with the percentage phagocytosis of apoptotic neutrophils. For this reason, preliminary experiments were performed to examine the uptake of erythrocytes that had been coated with different concentrations of IgG, and it was determined that opsonization with a 1:4000 dilution of antibody (polyclonal rabbit anti-human erythrocyte; Dako Ltd) resulted in phagocytosis of EIgG by approximately 30% of human macrophages. Previous studies have shown that a low-level opsonization strategy employed to coat erythrocytes with IgG resulted in a submaximal phagocytic response that could be enhanced by certain stimuli (for example, following adhesion of monocytes to fibronectin (Pommier et al., 1983)).

Incubation of macrophages with CD44 antibody had no demonstrable effect upon Fc receptor-mediated phagocytosis of EIgG (Figure 11). Similarly, there was no effect upon phagocytosis of zymosan, which is mediated by a combination of CD11b/CD18 ($\alpha_M\beta_2$; CR3), the mannose receptor, and the β-glucan receptor (Riches et al., 1988). Furthermore, there was no significant phagocytosis of neutrophils that had been freshly isolated from peripheral blood (>99% non-apoptotic) (Hart et al., 1997). These results provide evidence against a general effect of CD44 antibody upon macrophage phagocytic capability, and suggest that CD44 ligation has a specific effect on the putative phagocyte machinery that is involved in the binding and/or engulfment of apoptotic cells.

It has been proposed that the surface receptor utilised by phagocytes for recognition of apoptotic cells is independent of the lineage of the apoptotic target particle, and that the large number of different molecules implicated in apoptotic cell recognition is a function solely of the different lineages of phagocytes (Fadok et al., 1992a). If this were truly the case then CD44 ligation should up-regulate phagocytosis of not only apoptotic neutrophils, but also of other apoptotic cells. To further explore the effect of CD44 ligation on macrophages it was decided to investigate whether phagocytosis of apoptotic lymphocytes was influenced by CD44 antibody.

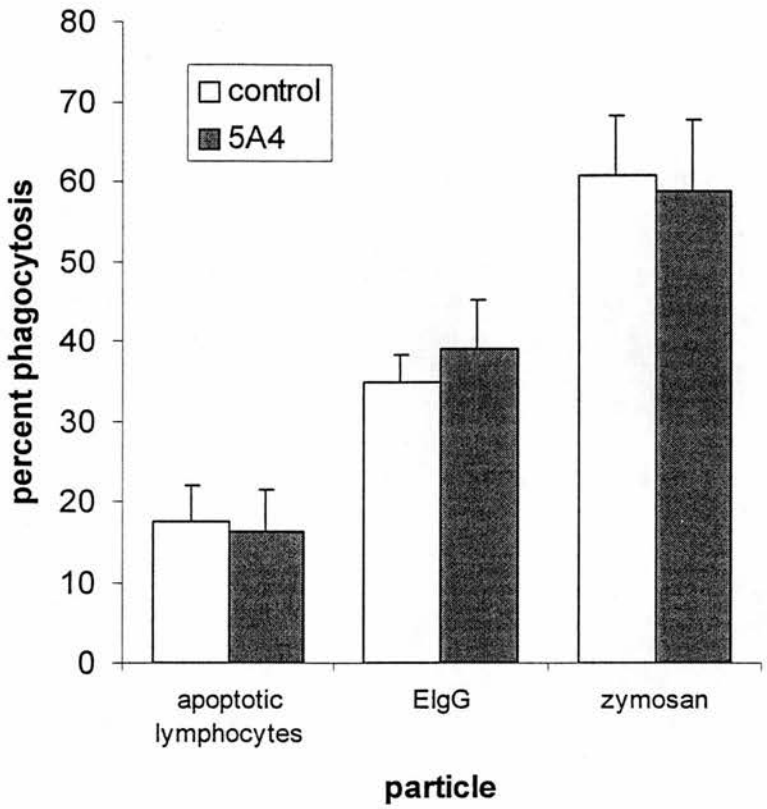


FIGURE 11.
Effect of CD44 antibody on macrophage phagocytosis is specific for apoptotic neutrophils.
Pre-treatment of macrophages with CD44 antibody 5A4 (shaded bars) had no effect upon phagocytosis of apoptotic peripheral blood lymphocytes, immunoglobulin G-opsonized erythrocytes (EIgG), or zymosan ($P>0.05$). Phagocytosis by control (untreated) macrophages is presented as open bars. n=3-5.

Phagocytosis of apoptotic lymphocytes

Lymphocytes were isolated from human peripheral blood and induced to undergo apoptosis by exposure to γ-irradiation. The standard phagocytosis assay for neutrophils had to be slightly modified since apoptotic lymphocytes do not contain myeloperoxidase and cannot be simply selectively stained in the plastic wells. To circumvent this problem macrophages were detached from the wells with trypsin-EDTA following the phagocytic interaction and cytocentrifuged onto glass slides before staining and counting. Apoptotic lymphocytes could be clearly visualised within phagocytic vacuoles of macrophages (figure 12). This method has previously been validated against the standard "in-well" counting technique for assessing CD44-augmented phagocytosis of apoptotic neutrophils (table III).

Interestingly, CD44 antibody had no effect upon either the proportion of macrophages that phagocytosed apoptotic peripheral blood lymphocytes (Figure 11,12), or the average number of apoptotic lymphocytes per phagocytosing macrophage (1.36±0.1 (control) vs. 1.34±0.1 (5A4); $P=0.56$; n=5). This finding is of interest in view of previously published observations suggesting that the nature of the apoptotic cell contributes little or nothing to the mechanism employed for its recognition by phagocytes

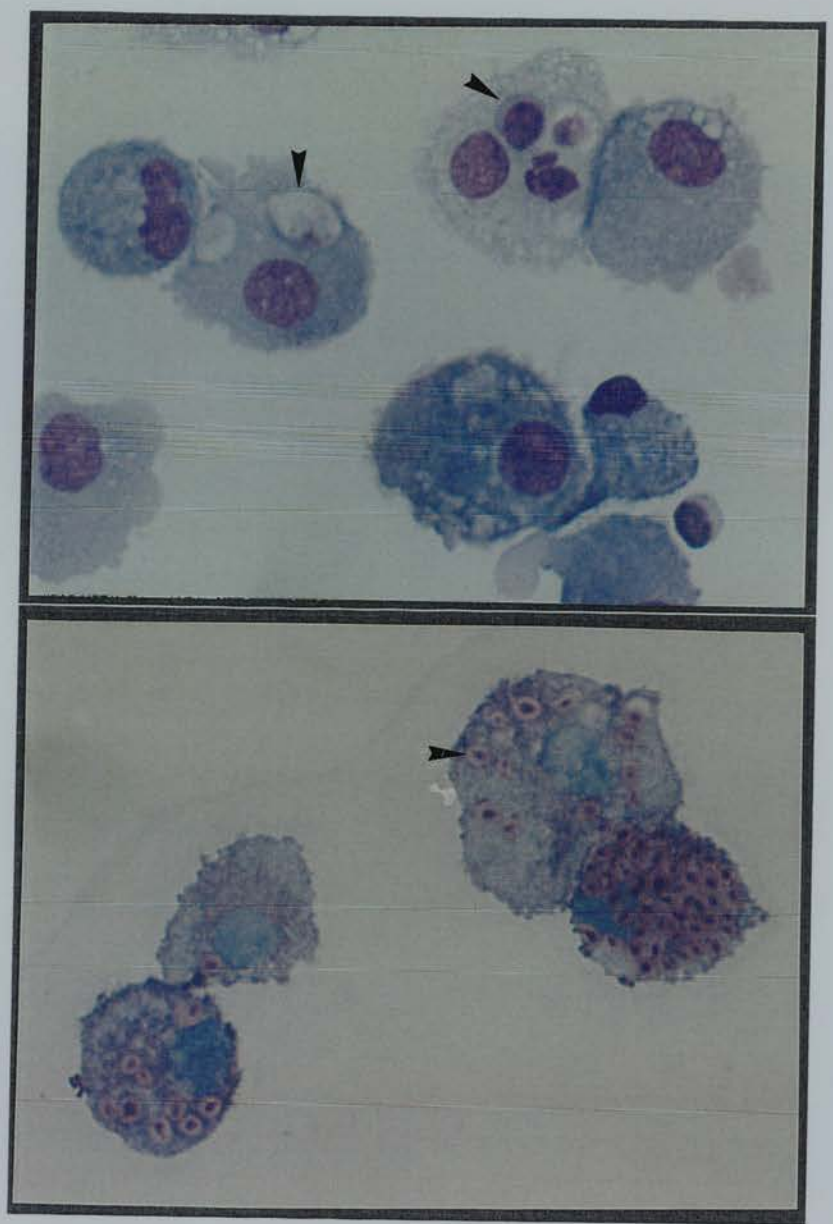


FIGURE 12

Macrophage phagocytosis of apoptotic lymphocytes and zymosan

Adherent human monocyte-derived macrophages were incubated with suspensions of apoptotic peripheral blood lymphocytes (upper panel) or zymosan particles (lower panel). Macrophages were then washed vigorously to remove non-ingested particles, detached from the plastic wells with trypsin-EDTA, and cytocentrifuged onto glass slides. Staining was with Diff-Quik (plus periodic acid-Schiff for zymosan, which stains the yeast cell wall pink). Ingested apoptotic lymphocytes and zymosan particles can be visualised within the macrophages.

(Fadok et al., 1992a). These results indicate that apoptosis may be associated with expression of cell-specific markers that signal recognition and phagocytosis by macrophages. This has important implications for the clearance of inflammatory cells during resolution of inflammation, suggesting that phagocytosis of potentially destructive apoptotic cells such as neutrophils may be specifically promoted following engagement of macrophage CD44. It would be of interest to determine whether CD44 antibody differentially altered macrophage phagocytosis of specific T lymphocyte subsets, or other apoptotic cell populations (e.g., NK cells) with the potential for induction of tissue damage if allowed to proceed through apoptosis to secondary necrosis.

SUMMARY

Phagocytosis of apoptotic neutrophil granulocytes by macrophages at inflammatory sites is an important determinant of the process by which inflammation resolves. Here it has been demonstrated that phagocytosis of apoptotic neutrophils, but not apoptotic lymphocytes, by human monocyte-derived macrophages is rapidly augmented following ligation of CD44 by bivalent antibodies in vitro. These observations, together with the lack of effect of CD44 antibodies upon macrophage phagocytosis of zymosan or immunoglobulin-opsonized erythrocytes imply that CD44 may regulate the differential clearance of apoptotic leukocytes during evolution of inflammatory responses, and suggest that unique molecular recognition pathways may be involved. This represents a novel role for CD44 in inflammation and tissue repair.

CHAPTER 4
MECHANISMS OF CD44-AUGMENTED PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS

INTRODUCTION

The previous chapter described how ligation of human macrophage CD44 by bivalent antibodies rapidly and greatly enhanced phagocytosis of apoptotic neutrophils in an in vitro assay. This novel finding raised the possibility that ligation of CD44 might be an important control mechanism for the clearance of apoptotic inflammatory cells during resolution of inflammation. It was therefore important to determine the mechanism(s) by which this phenomenon occurred. Data presented in the previous chapter demonstrated that CD44 ligation specifically augmented phagocytosis of apoptotic neutrophils, but not IgG-opsonized erythrocytes or zymosan yeast cell wall particles, and so a logical first step was to investigate the role of phagocyte receptors that have been implicated in apoptotic cell recognition. A number of molecules on the phagocyte surface have been proposed to directly mediate recognition and phagocytosis of apoptotic cells, including lectins, the integrin $\alpha_v\beta_3$ (CD51/61), CD36, a phosphatidylserine receptor, a scavenger receptor, and the 61D3 epitope of CD14 (Savill et al., 1993; Hart et al., 1996; Devitt et al., 1998). The molecular alterations on the surface of the apoptotic neutrophil that are responsible for phagocyte recognition have yet to be characterised. Much attention has been directed towards the anionic phospholipid phosphatidylserine (PS) (Verhoven et al., 1995), which is relocated from the inner to the outer surface of the plasma membrane during apoptosis (Homburg et al., 1995). PS exposure has been implicated in the recognition of apoptotic leukocytes by mouse inflammatory macrophages in vitro (Fadok et al., 1992b), but PS does not appear to be involved in apoptotic cell recognition by other phagocytes, including human macrophages (Fadok et al., 1992a; Hart et al., 1997). Considering the diversity of phagocyte surface molecules that have been proposed to be involved in the recognition of apoptotic cells it is extremely likely that additional undefined molecular changes on the apoptotic cell surface contribute to phagocyte recognition under different circumstances.

Inhibitors of putative phagocyte receptors for apoptotic cells have been well characterised (Savill et al., 1993). One strategy for definition of molecular pathways involved in apoptotic cell recognition would be to incubate a number of these previously defined ligands and antibodies with the macrophage monolayers following treatment with CD44 antibody. Natural ligands such as peptides, sugars, and lipids may bind with low affinity and are therefore used at high concentrations, although even then they may be unable to compete with surface bound ligands. Monoclonal antibodies may bind with very high affinity, but interpretation of their effects may be difficult because receptor crosslinking by bivalent (e.g., IgG) or multivalent (e.g., IgM) antibodies may exert regulatory effects on living cells. It was anticipated that a complementary approach using ligands and monoclonal antibodies may allow the molecular mechanism responsible for augmented phagocytosis of apoptotic neutrophils to be established.

RESULTS AND DISCUSSION

Role of previously defined macrophage molecules in CD44-augmented phagocytosis

A panel of ligands and antibodies that bind to relevant macrophage surface receptors was used to investigate whether CD44 ligation recruited previously defined mechanisms to augment phagocytosis of apoptotic neutrophils. However, none of the agents tested had any demonstrable inhibitory effect upon CD44-augmented phagocytosis of apoptotic neutrophils when used alone (table I), although all were used at concentrations that have previously been shown to inhibit binding or phagocytosis of apoptotic cells (Savill et al., 1993). The lack of inhibition observed with any of these agents when used singly suggests that ligation of macrophage CD44 either recruits multiple apoptotic cell recognition pathways simultaneously or brings into play novel phagocytic receptors.

Inhibitor of apoptotic cell recognition	**Mean percentage of CD44-augmented phagocytosis ± SEM (n)**
Monoclonal antibodies (specificity; concentration)	
CLB-IVC7 (CD36; 10μg/ml)	106 ± 16.4 (3)
61D3 (CD14; 1:100 ascites)	119 ± 6.6 (5)
Saccharides/glycoproteins (specificity; concentration)	
N-acetyl-D-galactosamine (ASGPR; 100 mM)	99.6 ± 7.6 (4)
N-acetyl-D-glucosamine (lectin; 10mM)	106 ± 10.6 (4)
dextran sulphate (scavenger receptor; 500μg/ml)	110 ± 15.7 (4)
fucoidan (scavenger receptor; 500 μg/ml)	92.1 ± 8.0 (3)
galactose (ASGPR; 50 mM)	103 ± 6.9 (4)
D-glucosamine ($\alpha_v\beta_3$/CD51/61; 10mM)	101 ± 5.9 (7)
mannan (mannose receptor; 1 mg/ml)	102 ± 6.8 (3)
Phospholipid derivatives (concentration)	
phospho-L-serine (PS receptor; 1 mM)	99.3 ± 4.6 (7)

TABLE I.
The lack of effect of previously defined inhibitors of apoptotic cell recognition on CD44-augmented phagocytosis of apoptotic neutrophils by human macrophages.
Inhibitors at the concentrations indicated were pre-incubated with the macrophage monolayer (antibodies) or added to the macrophage-aged neutrophil phagocytosis assay (sugars and phospholipids) following stimulation of macrophages with CD44 antibody 5A4. (ASGPR, asialoglycoprotein receptor).

Other candidate receptors: sialoadhesin

Sialoadhesin is a murine macrophage-restricted immunoglobulin superfamily receptor that binds to specific sialated sugars. It has been proposed that sialoadhesin mediates interactions between macrophages and developing myeloid cells in the bone marrow (Kelm et al., 1994). Ligand binding by sialoadhesin is independent of divalent cations, and so was a potential candidate for a surface molecule involved in CD44-augmented phagocytosis of apoptotic neutrophils (see later). However, when used at a

concentration known to block sialoadhesin-mediated adhesion the soluble sialated sialoadhesin ligand disialoganglioside GD1a (Kelm et al., 1994) had no effect on CD44-augmented phagocytosis of apoptotic neutrophils (table II). It is difficult to discount a role for sialoadhesin on the basis of lack of effect of a single ligand, but until human sialoadhesin is characterised and specific monoclonal antibodies become available the potential for further investigation at the present time is limited.

Role of complement

Both macrophages and neutrophils are routinely cultured in the presence of 10% autologous serum, but the macrophage-neutrophil phagocytosis assay is deliberately performed in the absence of serum to minimise possible interference from serum-derived immunoglobulins or complement components. However, in a serum free in vitro assay of phagocytosis of zymosan secretion of complement by macrophages may have lead to opsonization of the yeast particles that promoted their ingestion (Ezekowitz et al., 1985). Opsonization of apoptotic neutrophils by serum- or macrophage-derived complement components could feasibly mediate recognition via macrophage complement receptors. To investigate the role of complement in CD44-augmented phagocytosis of apoptotic neutrophils the anti-complement protein cobra venom factor (CVF) was pre-incubated with macrophages and also added to the phagocytosis assay. When used at a concentration that inhibits complement-mediated binding in a similar system (Bohnsack et al., 1985) CVF had no effect on CD44-augmented phagocytosis of apoptotic neutrophils (table II). An alternative approach to investigate the role of complement in this system would have been to block specific complement components or receptors with monoclonal antibodies. Experiments later in this chapter demonstrate the lack of effect of β2 integrin (CD18) antibodies which block complement receptors CR3 (CD11b) and CR4 (CD11c). Since at least four different complement receptors are known to exist and to be expressed by macrophages (Ross, 1989) a definitive experiment in which all complement receptors were blocked would be difficult to perform. Furthermore, if multiple blocking antibodies were used concurrently interpretation would be complicated because of the likelihood of confounding crosslinking and Fc receptor-mediated effects (CR1, CD3, and CR4 are all expressed by neutrophils which could become opsonized by antibodies).

Heparin

Heparin has previously been shown to be a potent inhibitor of phagocytosis of apoptotic neutrophils by human monocyte-derived macrophages when used at a high concentration (Savill et al., 1989a), although the mechanism of this action has not been documented. In other systems, heparin blocks cell adhesion events mediated by cell surface heparan sulphate proteoglycans (Lindahl et al., 1994; Jackson, 1997). No significant effect was observed when heparin was added to the phagocytic interaction between CD44-stimulated macrophages and apoptotic neutrophils (table II). The concentration of heparin used (5 mg/ml) was comparable to that shown previously to almost completely block apoptotic neutrophil recognition (6.6 mg/ml; Savill et al., 1989a), suggesting that heparin sulphate proteoglycans are unlikely to play a role in CD44-augmented phagocytosis. This observation also provides further evidence that CD44 ligation results in recruitment of a unique apoptotic cell recognition pathway that is not operational under control conditions.

Inhibitor (specificity; concentration)	Mean percentage of CD44-augmented phagocytosis ± SEM (n)
cobra venom factor (complement; 10 μg/ml)	102 ± 19.6 (2)
disialoganglioside GD1a (sialoadhesin; 100 μg/ml)	94.5 ± 10.2 (4)
heparin (heparan sulphate proteoglycans; 5 mg/ml)	85.3 ± 6.8 (3)

TABLE II.
The lack of effect of inhibitors of putative apoptotic cell receptor mechanisms on CD44-augmented phagocytosis of apoptotic neutrophils.
The anti-complementary protein cobra venom factor (CVF), sialoadhesin ligand ganglioside GD1a, or heparin were added to the macrophage-aged neutrophil the phagocytosis assay following stimulation of macrophages with CD44 antibody 5A4.

Lack of effect of previously defined inhibitors of apoptotic cell recognition

In this series of experiments none of the inhibitors used had any demonstrable effect on CD44-augmented phagocytosis of apoptotic neutrophils, despite the fact that inhibitors were freshly made up and used at concentrations that have previously been shown to significantly inhibit apoptotic cell recognition in other systems. One limitation of apoptotic cell recognition studies is the lack of a suitable "positive control" for many of the inhibitors. It should also be pointed out that the concentrations used may themselves be hugely in excess of those required to block receptor-ligand interactions. For example, fucoidan at a concentration of 50 μg/ml will completely inhibit binding of oxidised erythrocytes to resident mouse peritoneal macrophages (Sambrano et al., 1994), but a tenfold higher concentration was used to attempt to inhibit recognition of apoptotic neutrophils. Furthermore, many of the inhibitors are non-specific. For example, fucoidan blocks both scavenger receptor- and selectin-ligand binding (Varki, 1997).

Despite the limitations of these experiments, it was not possible to demonstrate the involvement of any of the "classical" apoptotic cell recognition molecules in CD44-augmented phagocytosis. Therefore, in order to characterise the fundamental characteristics of the receptor-ligand pathways that may be involved, the requirement for divalent cations and the sensitivity of the phagocyte or apoptotic cell to enzyme treatment was examined. It was hoped that these rather crude experiments would provide some basic information that would permit further studies to focus on the specific surface molecules involved.

Requirement for divalent cations

Many surface receptors exhibit divalent cation-dependent ligand binding (e.g., the integrins and selectins), whereas others demonstrate binding that is independent of cations (e.g., CD44, scavenger receptors). It was hoped that analysis of the requirements of the interaction between CD44-stimulated macrophages and apoptotic neutrophils for divalent cations might yield useful mechanistic information. By performing the phagocytosis assay in medium without divalent cations the differential requirement could be examined by

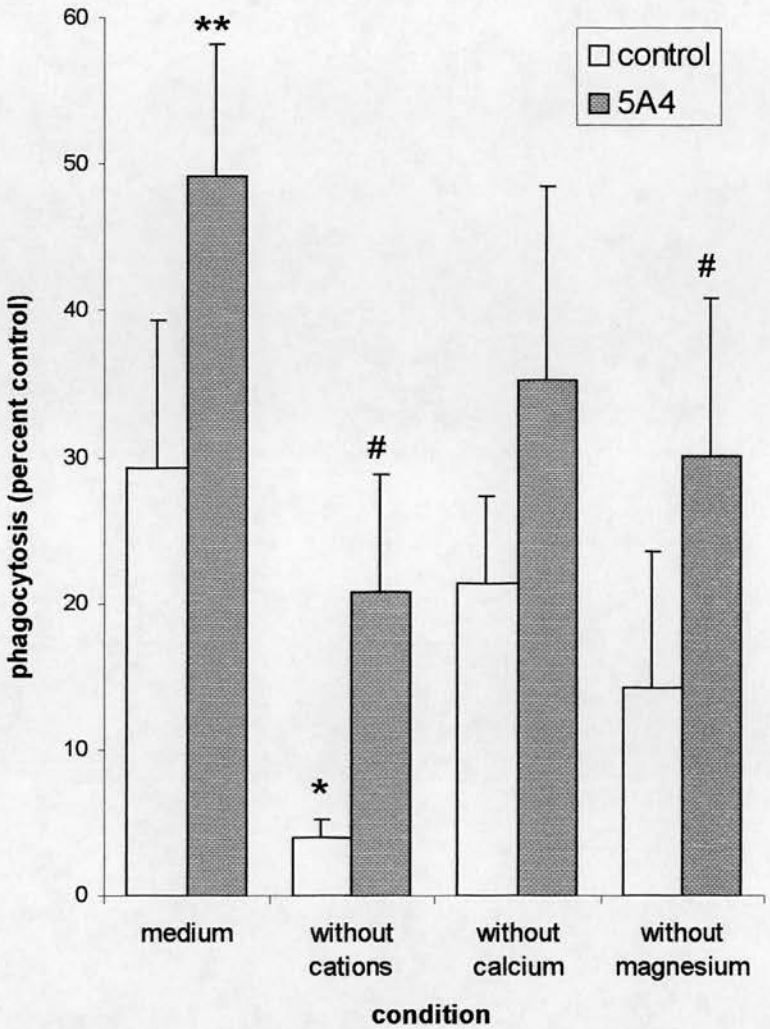


FIGURE 1

Requirement of CD44-augmented phagocytosis for divalent cations

Human monocyte-derived macrophages were incubated for 20 minutes with CD44 antibody 5A4 in Iscove's medium, and then washed twice in CMF-PBS. Incubations with aged human neutrophils were carried out for 30 minutes at 37°C in Iscove's medium, Iscove's medium without divalent cations as supplied by the manufacturer (Gibco), or cation-free Iscove's medium supplemented with 1.5 mM calcium chloride or 1.5 mM magnesium sulphate. Assessment of phagocytosis was carried out as previously described (n=3). * $P<0.05$, ** $P<0.01$ compared with control; # $P<0.05$ compared with 5A4.

selectively adding back calcium or magnesium. In the absence of CD44 antibody, macrophage phagocytosis of apoptotic neutrophils was inhibited to 15% of basal levels in the absence of divalent cations, and was restored to 40% and 99% of basal values by the addition of 1.5 mM calcium chloride or 1.5 mM magnesium sulphate respectively (figure 1). In contrast, removal of divalent cations resulted in a lesser inhibition of CD44-augmented phagocytosis (to 50% of the basal level), suggesting that CD44 ligation may have recruited a partly divalent cation-independent pathway for recognition of apoptotic neutrophils. Surprisingly, addition of calcium chloride or magnesium sulphate had little further effect (61% and 72% of basal levels respectively) (Figure 1).

Although the standard errors of the observations were relatively wide, these experiments clearly demonstrate that untreated and CD44 antibody-treated macrophages exhibit different patterns of divalent cation dependency for phagocytosis of apoptotic neutrophils. These data support the suggestion that CD44 ligation results in recruitment of a different molecular mechanism for apoptotic neutrophil phagocytosis when compared with basal conditions.

Enzyme treatment of the macrophage surface

As an alternative approach to investigate the molecular mechanisms of the macrophage receptors that may be playing a role in CD44-augmented phagocytosis, macrophages or aged neutrophils were treated with different enzymes prior to the phagocytic assay (Bazil, 1995).

Macrophage CD44 is sensitive to cleavage by proteases, so that incubation of macrophages with trypsin results in diminished reactivity with CD44 antibodies such as 5A4 (35.3% of control levels following 500 μg/ml trypsin for 20 minutes at 37°C). For this study, macrophages were incubated with 5A4 for 20 minutes, washed, and then exposed to enzymes or medium alone for 30 minutes. The monolayer was then washed again and a suspension of aged neutrophils added for assessment of phagocytosis of apoptotic cells. By using such a protocol it was hoped that the effect of enzyme treatment on the surface receptors directly involved in apoptotic neutrophil recognition could be assessed following CD44 ligation by antibody.

Exposure of macrophages to any of the broad spectrum proteases trypsin, pronase, or proteinase K resulted in significant inhibition of both basal and CD44-stimulated phagocytosis of apoptotic cells (figure 2). Treatment with these concentrations of enzyme had no effect on cell viability or cell adhesion as assessed by the number of macrophages remaining adherent to the plastic wells following the phagocytosis assay. In contrast, incubation with phosphatidylinositol specific phospholipase C (PI-PLC), which cleaves GPI-linked surface proteins, had no significant effect on basal or CD44-augmented phagocytosis of apoptotic neutrophils. The concentration of PI-PLC used was shown to induce maximal (approximately 50%) cleavage of CD14 in flow cytometric studies.

Although there was a little variation in the effect of the different proteases on phagocytosis of apoptotic neutrophils, it is clear from this experiment that macrophages utilise a protease-sensitive mechanism for recognition of apoptotic cells under both control conditions and following CD44 ligation. However, there was no significant differential effect on basal and CD44-augmented phagocytosis. Consequently, from the result of this experiment it was not possible to determine whether or not control and CD44-augmented phagocytosis were mediated by different surface recognition molecules. It remains possible that more sensitive experiments comparing protease concentration-response curves could reveal a difference in protease sensitivity between basal and CD44-augmented conditions.

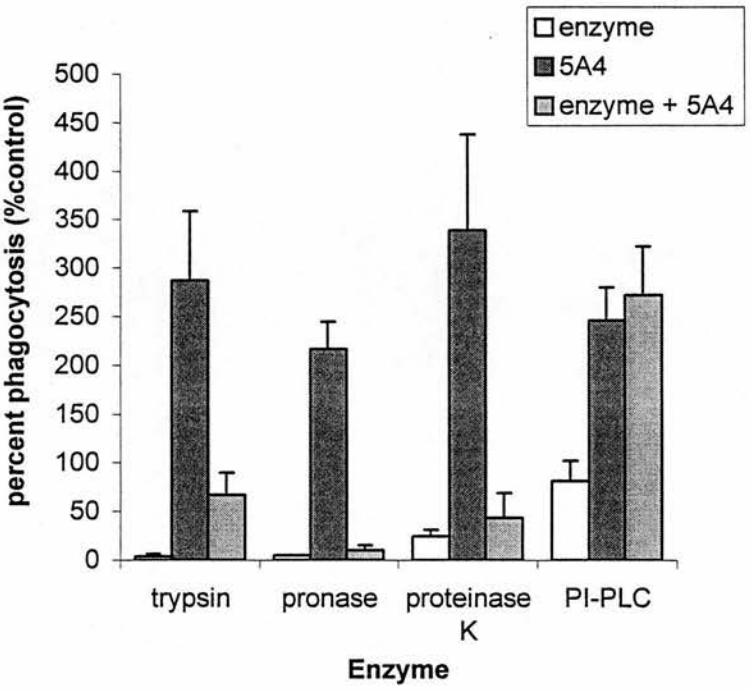


FIGURE 2
Effect of enzyme treatment of the macrophage surface on phagocytosis of apoptotic neutrophils
Human monocyte-derived macrophages adherent to 48 well plates were incubated for 20 minutes at room temperature with antibody 5A4 (1:5 supernatant) or medium alone, washed, and incubated for 30 minutes at 37°C with 100 μg/ml trypsin type IX (from porcine pancreas), 100 μg/ml pronase, 100 μg/ml proteinase K, or 0.5 U/ml phosphatidylinositol specific phospholipase C (PI-PLC). Phagocytosis of apoptotic neutrophils was then assessed. To account for inter-experiment variability in the basal rate of phagocytosis results have been expressed as percentage of control, and the mean ± SEM of three independent experiments is presented.

Effect of treatment of the neutrophil surface with enzymes

In contrast to their effect when incubated with macrophages, broad spectrum proteases had little effect on phagocytosis of apoptotic neutrophils when they were pre-incubated with the target particles (figure 3). Enzyme treatment was shown to be effective in that it cleaved protease-sensitive molecules (such as CD14 and CD44) from the neutrophil surface in flow cytometric studies. These results imply that, unlike the protease-sensitive macrophage apoptotic cell receptors, the apoptotic cell ligands that are recognised by phagocytes are relatively protease-resistant.

Treatment of the aged neutrophil surface with neuraminidase significantly enhanced the basal level of apoptotic neutrophil phagocytosis (figure 3). The significance of this observation is examined further when the plasma membrane changes associated with apoptosis are considered in chapter 5.

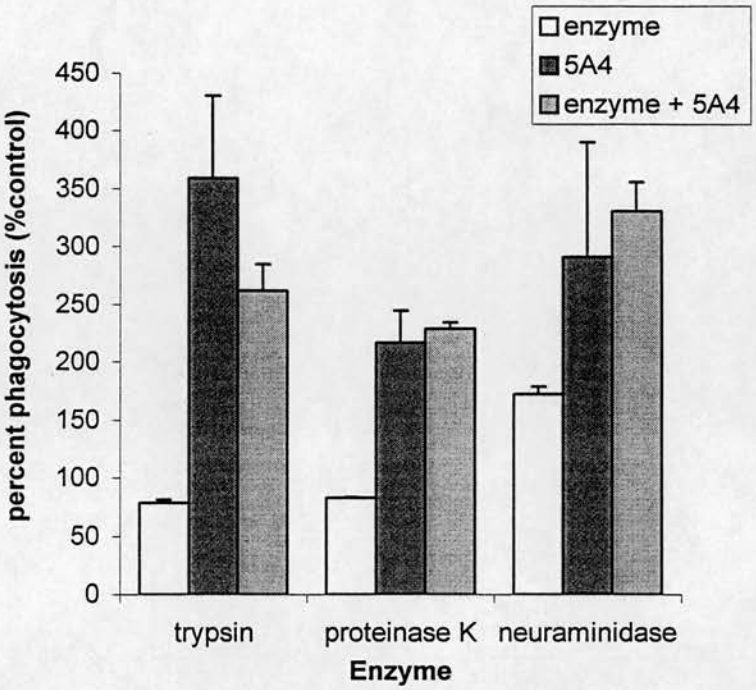


FIGURE 3

Effect of enzyme treatment of the aged neutrophil surface on phagocytosis of apoptotic neutrophils

Human monocyte-derived macrophages adherent to 48 well plates were incubated for 20 minutes at room temperature with antibody 5A4 (1:5 supernatant) or medium alone. Twenty-hour aged human peripheral blood neutrophils were incubated for 30 minutes at 37°C with 100 μg/ml trypsin type IX (from porcine pancreas), 100 μg/ml proteinase K, or 0.1 U/ml Vibrio cholerae neuraminidase. After washing the cells were then incubated with the macrophage monolayer and phagocytosis of apoptotic neutrophils was assessed. To account for inter-experiment variability in the basal rate of phagocytosis results have been expressed as percentage of control, and the mean ± SEM of three independent experiments is presented.

Augmented phagocytosis does not involve adhesion to hyaluronan

Binding of CD44 to cell surface hyaluronan has been directly implicated in a variety of cell adhesion processes (Green et al., 1988; Culty et al., 1990; Underhill, 1992; Lesley et al., 1993), and macrophages themselves may synthesize and secrete hyaluronan during in vitro culture (Laurent et al., 1996). It was hypothesised that a coating of hyaluronan on the macrophage surface may influence the interaction with apoptotic neutrophils, and that the augmentation of phagocytosis observed following binding of CD44 antibody may reflect disruption or enhancement of hyaluronan-mediated bridging of the macrophage and apoptotic cell membranes. However, experimental data suggested that CD44-hyaluronan interactions were unlikely to be involved in the augmentation of macrophage phagocytosis of apoptotic neutrophils. Augmented phagocytosis was unaffected by addition to the phagocytosis assay of high concentrations (100 μg/ml) of exogenous hyaluronan (table III). Similar concentrations have been shown to inhibit CD44-hyaluronan-mediated adhesion in other systems (Haegel et al., 1993; DeGrendele et al., 1996). Furthermore, incubation of macrophages or aged neutrophils with hyaluronidase had no demonstrable effect upon phagocytosis of apoptotic neutrophils (table III). However, this particular experiment was limited by the lack of a convenient positive control to prove that the enzyme was active.

Further evidence for the lack of a role for hyaluronan was provided by the observation that adhesion of macrophages to hyaluronan-coated plastic, which would be expected to redistribute CD44 and/or other hyaluronan receptors to the undersurface of the macrophage and so down-regulate receptor expression on the free surface, had no effect upon phagocytosis of apoptotic neutrophils (99.0±6.0% of control phagocytosis; P=0.87; n=2 (Hart et al., 1997)). These results argued against a direct role for CD44 in the interaction between macrophages and CD44, since on hyaluronan-coated plastic the number of CD44 molecules available to interact with apoptotic cells would be expected to be diminished. However, experiments to demonstrate down-regulation of free surface CD44, for example using immunocytochemistry with CD44 antibodies, were not performed here. Such experiments were used to support a role for the mannose receptor in binding and uptake of Pneumocystis carinii (Ezekowitz et al., 1991). Although receptor down-modulation experiments provide powerful evidence for the role of specific molecules in phagocytosis, it is interesting that they make virtually no contribution to published studies of the molecular mechanisms of phagocyte recognition of apoptotic cells.

The different effects upon CD44-hyaluronan binding of different CD44 antibodies provides additional evidence against a role for CD44-hyaluronan interactions in regulation of phagocytosis of apoptotic neutrophils. For example, CD44-mediated adhesion to hyaluronan is inhibited by antibodies 5A4, 3C9, and 3C12 (Dougherty et al., 1994; Droll et al., 1995), but is enhanced by F.10.44.2 (Liao et al., 1995). In contrast, all four of these pan-reactive CD44 antibodies promoted phagocytosis of apoptotic neutrophils to a similar extent (Hart et al., 1997).

Augmented phagocytosis is not mediated by β_2 integrins

Previous studies have demonstrated that ligation of surface CD44 by antibodies may induce cell adhesion via activation of β_2 integrins (Koopman et al., 1990; Bruynzeel et al., 1993; Vermot-Desroches et al., 1995). These observations raise the possibility that binding of CD44 antibody to macrophages may activate β_2 integrins to engage ligands (e.g., ICAM-3) present on the apoptotic neutrophil surface, resulting in tethering of the apoptotic target cells that subsequently leads to phagocytosis. However, a function-blocking β_2 integrin (CD18) antibody had no effect upon CD44-augmented phagocytosis of apoptotic neutrophils when pre-incubated with macrophages and included in the phagocytosis assay (table III; Hart et al., 1997). These data indicate that rapid augmentation of phagocytosis of apoptotic neutrophils following binding of CD44 antibody to macrophages is likely to be independent of β_2 integrin-mediated adhesion.

Treatment	Mean percentage of CD44-augmented phagocytosis ± SEM (n)
TS1-18 (β2 integrin/CD18; 20μg/ml) [1]	104 ± 7.1 (5)
hyaluronan (from Sigma; 100μg/ml) [2]	90.1 ± 5.5 (3)
Streptomyces hyaluronidase (20 U/ml) on aged neutrophils [1]	108 ± 1.5 (3)
Streptomyces hyaluronidase (20 U/ml) on macrophages [1]	82 (1)

TABLE III.
The lack of effect of potential inhibitors on CD44-augmented phagocytosis of apoptotic neutrophils.
Agents were pre-incubated with cells [1] or included in the phagocytosis assay [2].

Cytoskeletal inhibitors

The process of binding and then phagocytosis of a target particle such as an apoptotic neutrophil is dependent upon complex rearrangements of the macrophage cytoskeleton and extension of cell processes (Brown, 1995). Interestingly, the C. elegans gene product ced-5, which is required for the orderly removal of cellular corpses in the nematode, resembles human DOCK180, a cytoskeletal linker protein implicated in regulation of cell morphology (Hasegawa et al., 1996; Wu and Horvitz, 1998). It has been shown that phagocytosis mediated by different membrane receptor pathways, namely receptors for Ig-Fc and complement, exhibit differential sensitivity to inhibitors of cytoskeletal function (Newman et al., 1991). For example, uptake of complement-opsonized sheep erythrocytes by human macrophages was blocked by both cytochalasin B, a microfilament inhibitor, and nocodazole, an inhibitor of microtubules. In contrast, uptake of Ig-opsonized erythrocytes was blocked only by cytochalasin, and was insensitive to nocodazole. CD44 associates via its cytoplasmic tail and transmembrane domain with cytoskeletal elements such as members of the ERM family of proteins (Tsukita et al., 1994; Isacke, 1994). Preliminary observations of macrophages stained with rhodamine-phalloidin following binding of CD44 antibodies hinted at subtle alterations of the actin cytoskeleton following CD44 ligation, although this proved very difficult to quantify (I. Dransfield, unpublished observations). It was hypothesised that augmented macrophage phagocytosis of apoptotic neutrophils following cross-linking of CD44 by bivalent antibodies may be the result of some alteration in the macrophage cytoskeleton. By using different cytoskeletal inhibitors in a similar manner to the experiments of Newman et al, the requirement for cytoskeletal components of CD44-augmented phagocytosis could be analysed.

Human monocyte-derived macrophages were incubated with cytochalasin B, nocodazole, or colchicine (another microtubule inhibitor) and the effect on phagocytosis apoptotic neutrophils with or without pre-incubation with CD44 antibodies was observed. It was noted that all of these agents induced profound changes in macrophage morphology. Cytochalasin induced cell rounding without detachment, whereas colchicine and nocodazole resulted in retraction, spiculation, and some detachment. However, there was

no differential effect of the cytoskeletal inhibitors on control- or CD44-augmented phagocytosis of apoptotic neutrophils, which was significantly inhibited by all treatments (figure 4). The conclusion from these experiments is that microtubules and microfilaments are both essential for human macrophage phagocytosis of apoptotic neutrophils under basal conditions and following ligation of CD44. There was again no intimation from these studies as to the mechanism of CD44-augmented phagocytosis of apoptotic neutrophils.

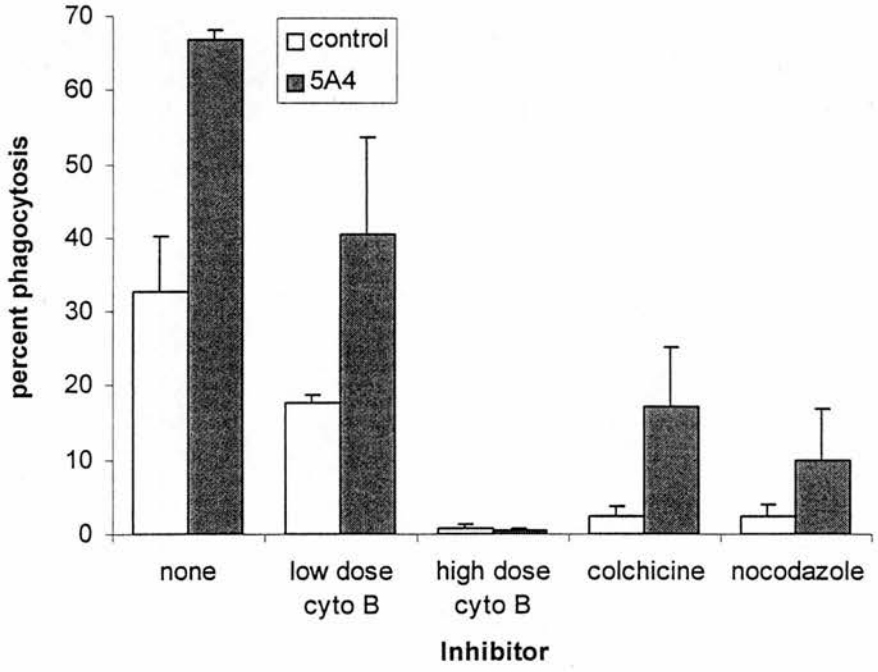


FIGURE 4
Role of cytoskeletal elements in CD44-augmented phagocytosis of apoptotic neutrophils
Human monocyte-derived macrophages were stimulated with CD44 antibody 5A4 and then phagocytosis of apoptotic neutrophils was assessed in the presence of medium alone (no inhibitor), cytochalasin B 0.1 μg/ml (low dose cyto B), cytochalasin B 5 μg/ml (high dose cyto B), colchicine 10 μg/ml, or nocodazole 2.5 μg/ml. Results are presented as mean ± SEM.

Other considerations: Role of cytokine release

Ligation of CD44 by antibodies may stimulate release of pro-inflammatory cytokines, such as TNF-α (Webb et al., 1990) or M-CSF (Noble et al., 1993), from human monocytes, although no such effects have been reported in macrophages. A variety of pro-inflammatory cytokines have been proposed to promote phagocytosis of apoptotic cells in vitro (Ren and Savill, 1995). However, this effect required exposure of macrophages to pro-inflammatory cytokines for a minimum of four hours, suggesting that synthesis of new protein(s) was required. In contrast, CD44-mediated promotion of phagocytosis of apoptotic neutrophils occurred rapidly (within minutes), suggesting that this effect was independent of macrophage cytokine production. Furthermore, no release of the pro-inflammatory cytokines TNF-α or IL-8 could be demonstrated following binding of CD44 antibodies to human monocyte-derived macrophages (chapter 4).

Role of intercellular bridging by antibody

When macrophages were pre-incubated with proteolytic fragments of CD44 antibody 5A4 augmented phagocytosis of apoptotic neutrophils was observed only with $F(ab')_2$ fragments, and not with Fab' fragments (chapter 3). These results discount a role for binding of CD44 antibody to macrophage Fc receptors in the augmentation of phagocytosis. However, one unlikely explanation for these findings was that bivalent CD44 antibody had acted as a "bridge" between the macrophage and the apoptotic neutrophil, resulting in increased binding between the two cell types. Several lines of evidence argue against this possibility. First, CD44 antibody-treated macrophages did not bind freshly isolated (non-apoptotic) neutrophils (Hart et al., 1997), despite high levels of neutrophil surface expression of CD44. Indeed, dual label flow cytometric analysis demonstrated that CD44 expression was lower on apoptotic neutrophils than on non-apoptotic neutrophils (mean relative expression 64±3.4%; n=3). Second, no binding of apoptotic cells was observed when CD44 antibody-treated macrophages were incubated with apoptotic neutrophils at 4°C (I. Dransfield, unpublished observations). Third, other antibodies recognising abundant antigens expressed by macrophages and neutrophils failed to augment phagocytosis (e.g., antibody W6/32; chapter 3). Together, these data demonstrate that bivalent CD44 antibody had not simply acted as a bridge between the macrophage and the apoptotic neutrophil.

SUMMARY

In this chapter I have started to define how CD44 ligation promoted human macrophage phagocytosis of apoptotic neutrophils. The data presented here suggest that rather than simply up-regulating the basal apoptotic cell recognition mechanism, CD44 ligation results in recruitment of a new molecular mechanism that is much less dependent on the presence of divalent cations than the basal pathway. None of a number of previously defined inhibitors of apoptotic cell recognition had any effect on CD44-augmented phagocytosis, suggesting that classical apoptotic cell recognition receptors such as $\alpha v\beta 3$, lectins, the phosphatidylserine receptor, scavenger receptor, of the 61D3 epitope of CD14 were not involved. In addition, I have used monoclonal antibodies and competitive ligands to argue against a role for other cell adhesion molecules such as $\beta 2$ integrins or sialoadhesin. The apoptotic cell receptor mechanism recruited following CD44 ligation was, like the basal recognition mechanism, sensitive to broad spectrum proteases and cytoskeletal inhibitors, but did not appear to be GPI-linked to the macrophage membrane.

Identification of the apoptotic cell recognition receptor(s) activated by CD44 ligation may prove difficult. A role for individual candidate receptors can be ruled out by examining the effect of inhibitory ligand or antibodies in the phagocytosis assay. However, a screening approach such as this will be ineffective if a previously uncharacterised receptor (or combination of receptors) is involved. Perhaps a more fruitful way of tackling this question would be to examine the changes that occur in the macrophage following binding of CD44 antibody and the functional response of macrophages to ingestion of apoptotic neutrophils. These issues are examined in the next chapter.

MACROPHAGE CYTOKINE RELEASE IN RESPONSE TO PHAGOCYTOSIS OF APOPTOTIC NEUTROPHILS

INTRODUCTION

As part of the host defence against invading micro-organisms, mononuclear cells (such as macrophages) and granulocytes are armed with the capability to ingest particulate matter prior to intracellular killing. Phagocytosis of micro-organisms by leukocytes occurs most efficiently in the presence of serum factors termed opsonins, which bind to and coat the microbes (Heijnen and van de Winkel, 1997). The principal opsonins are IgG (mainly IgG_1 and IgG_3 in humans) and complement components C3b and iC3b. Following opsonisation of the microbe, engulfment occurs following ligation of Fc receptors (three related molecules of the Ig superfamily CD16, CD32, and CD64) or complement receptors (CD35, CD11b/CD18, CD11c/CD18) on the phagocyte surface membrane (Heijnen and van de Winkel, 1997). In addition to promoting recognition and phagocytosis of pathogenic organisms, opsonization by IgG activates leukocyte Fcγ receptors, resulting in transduction of intracellular signals and subsequently metabolic enhancements that increase the efficiency of killing of the target particles (Allen and Aderem, 1996a; Sanchez-Mejorada and Rosales, 1998). Perhaps one of the best characterised examples of Fc receptor-mediated signalling occurs in NK cells. Recognition of IgG-opsonized particles by the NK cell low affinity receptor FcγRIIIA (CD16) activates the NK cell to secrete inflammatory cytokines such as TNF-α and IFN-γ and to discharge their granules, which may mediate cytolysis of the target cell (Sanchez-Mejorada and Rosales, 1998).

In vitro, phagocytosis of IgG-opsonized particles stimulates the macrophage to synthesize and release inflammatory mediators such as enzymes, arachidonic acid metabolites, and cytokines (Dean et al., 1979; Debets et al., 1988; Ida et al., 1988). Acquisition of a "pro-inflammatory" macrophage phenotype serves to propagate the host's defence to invading micro-organisms. Similarly, so called non-specific phagocytosis, in which ingestion of particles such as latex beads or aldehyde-fixed erythrocytes occurs in the absence of identified receptor-ligand interactions (Benoliel et al., 1980), may induce cytokine release by the phagocyte (Meagher et al., 1992). In contrast, phagocytosis of apoptotic cells has been assumed to represent an anti-inflammatory process and has been reported not to induce, or even to inhibit pro-inflammatory mediator release from macrophages (Meagher et al., 1992; Fadok et al., 1998).

Ligation of macrophage CD44 with bivalent antibodies markedly augments macrophage phagocytosis of apoptotic neutrophils, and it has been hypothesised that this could prove to be a useful means of promoting resolution of inflammation as part of a potential therapeutic strategy for inflammatory disease (Haslett, 1997). It was therefore important to determine whether CD44-augmented phagocytosis of apoptotic neutrophils influenced macrophage release of inflammatory mediators. Previous studies of macrophage mediator production in response to apoptotic cell phagocytosis have used opsonized zymosan (incubated with human serum) as a control particle to judge the "maximal" level of macrophage mediator release (Meagher et al., 1992; Stern et al., 1996). However, there may be important differences between phagocytosis of opsonized zymosan and apoptotic neutrophils. Particles of zymosan (average diameter 2

μm) are small compared with apoptotic neutrophils (6 μm), and are taken up much more avidly by human monocyte-derived macrophages, both in terms of the average number of particles per ingesting macrophage and the proportion of macrophages that engulf one or more particles. Indeed, analysis of macrophage cytocentrifuge preparation following ingestion of "unopsonized" zymosan indicated that some cells were so full of ingested particles that they had begun to rupture. For these reasons, IgG-opsonized erythrocytes were used as an additional control particle in this study, since erythrocytes are of similar size to apoptotic neutrophils and it has been established that the concentration of opsonizing antibody can be titrated to give similar rates of phagocytosis (chapter 3).

As an additional variable, macrophages were incubated with 50 μg/ml E. Coli 018 lipopolysaccharide (LPS) or medium alone for four hours prior to the phagocytosis assay. It has been reported that stimulation with LPS increases the basal level of inflammatory cytokine release (Fadok et al., 1998), so that any inhibition following phagocytosis of apoptotic neutrophils would be unmasked. The macrophage monolayer was then co-incubated with medium alone, apoptotic neutrophils, IgG-opsonized erythrocytes, or zymosan particles. Following a 60 minute phagocytosis assay the non-ingested particles were washed away as usual and the macrophages were incubated in medium alone in the absence of serum. Parallel wells were fixed in glutaraldehyde and subsequently stained with DMB/H_2O_2 for assessment of phagocytosis rates. At four and eighteen hours the supernatants were collected, cleared of contaminating cells by centrifugation, and stored at -20°C. Although many inflammatory mediators may be released by macrophages, in this study it was decided to measure TNF-α and IL-8 as two examples of pro-inflammatory cytokines.

RESULTS AND DISCUSSION

Confirmation of CD44-augmented phagocytosis

In the series of three independent experiments in which macrophage supernatants were harvested for measurement of cytokine concentrations, incubation with CD44 antibody 5A4 prior to the assay resulted in a 2.2-fold increase in the proportion of macrophages that ingested apoptotic neutrophils (table IV). Ingestion of IgG-opsonized erythrocytes occurred at a slightly lower rate than that of apoptotic neutrophils under basal conditions (table IV).

Condition	Mean percent phagocytosis ± SEM
Control PMN	22.4 ± 8.2
5A4 PMN	47.2 ± 7.9
EIgG	15.9 ± 6.7

TABLE IV

Assessment of phagocytosis in parallel with assays of cytokine release

Human monocyte-derived macrophages were incubated for 60 minutes with immunoglobulin-opsonized erythrocytes (EIgG) or apoptotic neutrophils (PMN) under control conditions and following CD44 ligation with antibody 5A4. After washing, supernatants were harvested for cytokine assays, and parallel wells were processed for assessment of phagocytosis following staining with 0.1 mg/ml DMB/ 0.03% hydrogen peroxide. Results are presented as mean percent phagocytosis ± SEM of three independent experiments.

Macrophage release of tumour necrosis factor-α

Basal release of TNF-α under these experimental conditions was undetectable, being below the lower limit of detection of this TNF ELISA (4.4 pg/ml) (figure 5). TNF-α production remained limited even after incubation with LPS, although a low concentration of LPS (50 ng/ml) was chosen that it was hoped would induce submaximal cytokine release and so allow the true effects of phagocytosis of different particles to be observed. Similarly, zymosan, which has been reported to be a potent stimulator or cytokine production in mononuclear phagocytes, failed to induce any significant TNF-α production by human macrophages in this series of experiments. This may be a reflection of the populations of cell used in these studies, since mature macrophages have greatly reduced secretory potential compared with the monocytes from which they are derived. However, other studies have reported significant measurable release of TNF-α from human macrophages in response to LPS or zymosan (Fadok et al., 1998).

Analysis of figure 5 reveals that the augmented phagocytosis of apoptotic neutrophils following CD44 ligation by antibody 5A4 had no significant effect on macrophage TNF-α release compared with that following basal phagocytosis of apoptotic neutrophils. However, the lack of stimulatory effect of LPS, EIgG, or zymosan is intriguing, and there are several possible explanations for this observation. Low concentrations of TNF-α in the macrophage supernatants may reflect genuinely low levels of cytokine secretion. Alternatively, released TNF-α could be sequestered by either specific TNF receptors or by surface carbohydrates on the macrophages (Nathan and Sporn, 1991). A further possibility is that TNF-α is bound to products in the supernatant, such as soluble TNF receptors cleaved from the surface of macrophages or apoptotic neutrophils, so making the TNF-α inaccessible to the antibodies in the ELISA.

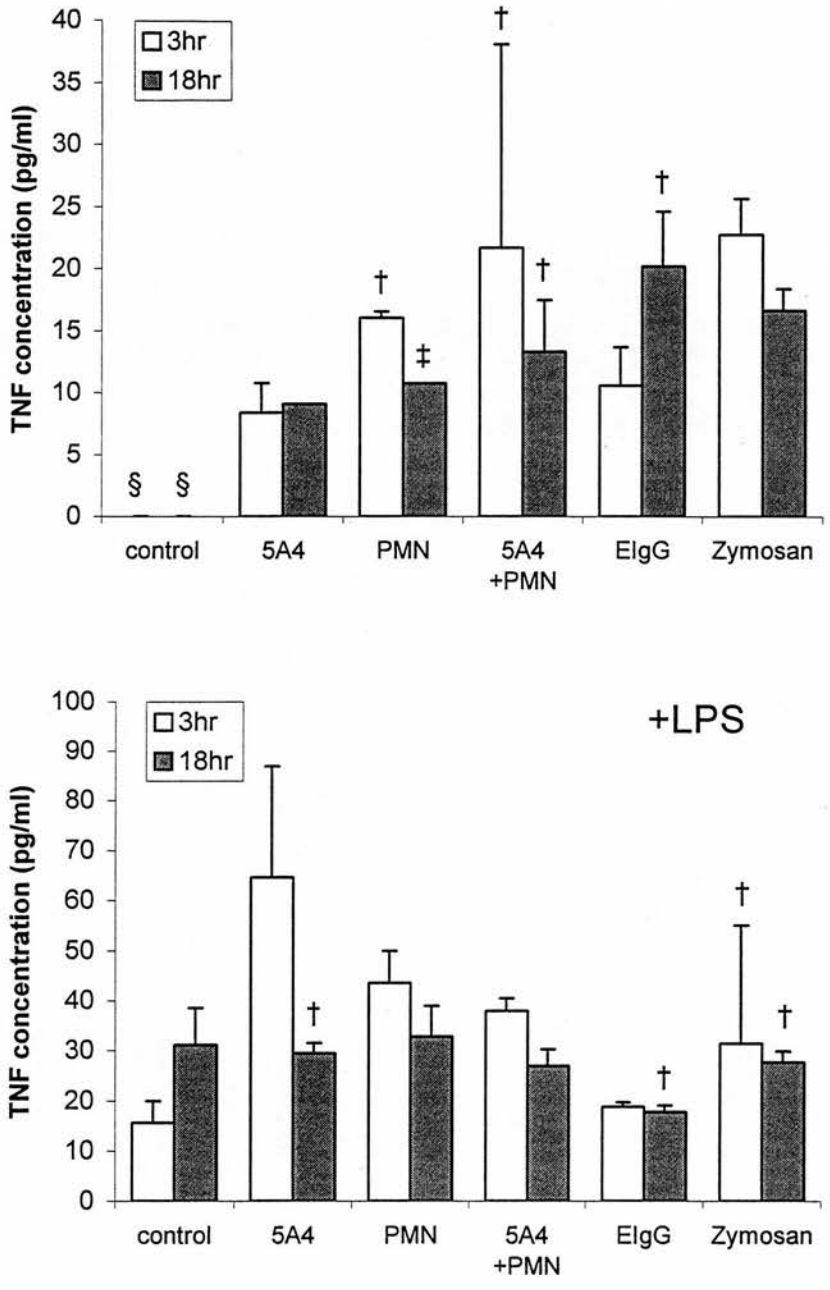


FIGURE 5

Release of TNF-α by macrophages in response to phagocytosis of apoptotic neutrophils

Human monocyte-derived macrophages were incubated for four hours in Iscove's medium alone (upper panel) or medium containing 50 ng/ml E. coli O18 lipopolysaccharide (lower panel). Cells were then washed three times prior to addition of CD44 antibody 5A4 or control for 30 minutes. After washing, macrophages were incubated at 37°C for 60 minutes with suspension of aged neutrophils (PMN), immunoglobulin G-opsonized erythrocytes (EIgG), zymosan, or medium alone (control). Non-ingested particles were washed away, and 0.5 ml of fresh serum-free medium was added. The medium was harvested at three hours, replaced, and then harvested again at 18 hours. Supernatants were centrifuged to remove cells and stored at –80°C prior to TNF-α ELISA. Results are presented as mean ± SEM of three independent experiments. For some conditions, one (†), two (‡), or all three (§) results were below the lower limit of detection of the assay (4.4 pg/ml). P>0.05 for all comparisons.

Macrophage release of interleukin-8

Because of the low basal and stimulated output of TNF-α from these human monocyte-derived macrophages, production of a second inflammatory cytokine, the principal neutrophil chemoattractant interleukin-8, was examined in the same samples.

In contrast to TNF-α, moderate amounts of IL-8 were found in the macrophage supernatants under basal conditions, and significant stimulation was observed following incubation with both LPS and zymosan (figure 6). It is clear that neither CD44 ligation alone, phagocytosis of apoptotic neutrophils, nor CD44-augmented phagocytosis had any significant effect on macrophage IL-8 release. Furthermore, although the rate of phagocytosis of EIgG was only one third that of apoptotic neutrophils following CD44-ligation, IL-8 release was significantly greater following incubation with EIgG.

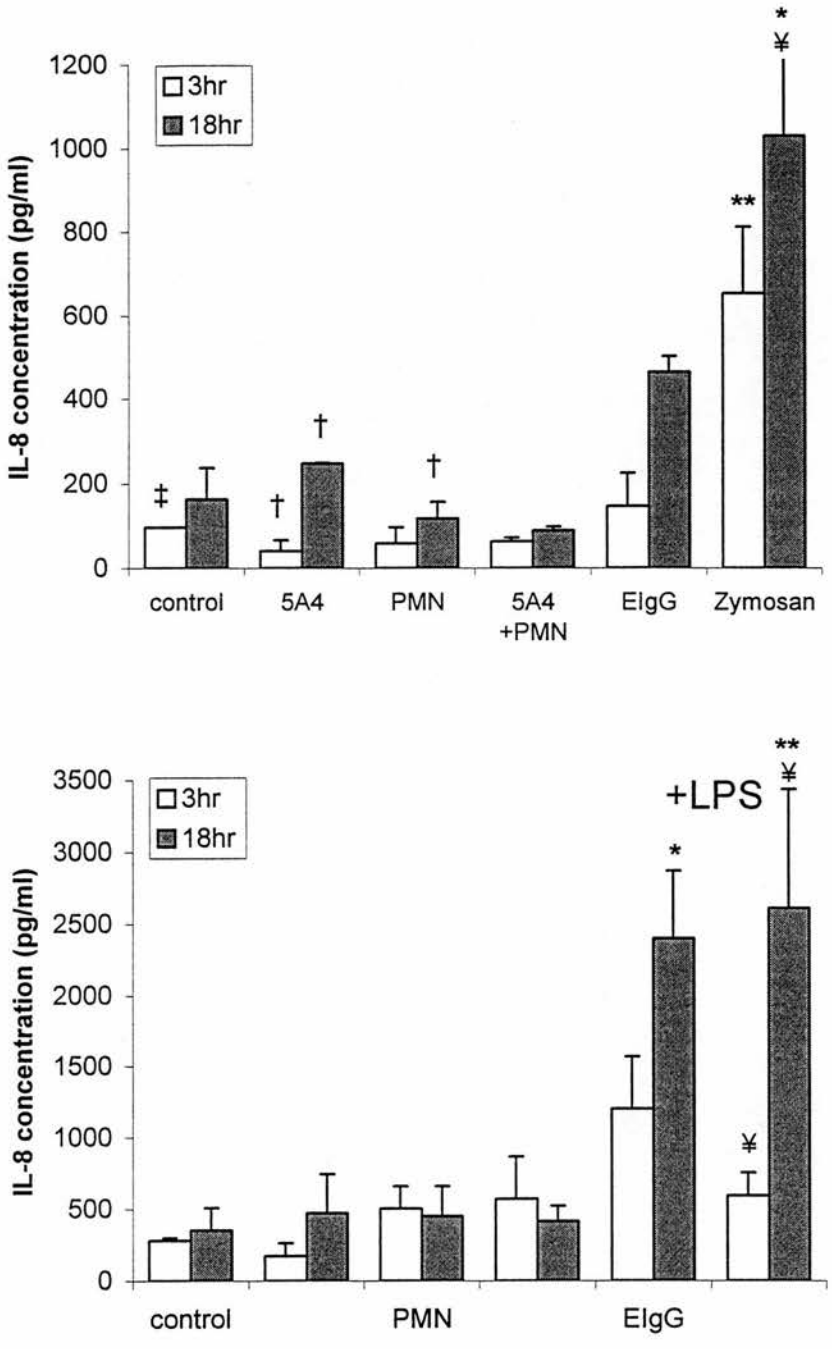


FIGURE 6

Release of IL-8 by macrophages in response to phagocytosis of apoptotic neutrophils

Human monocyte-derived macrophages were pre-incubated for four hours in medium alone (upper panel) or medium containing 50 ng/ml E. coli O18 LPS (lower panel). Cells were then washed three times prior to addition of CD44 antibody 5A4 or medium alone for 30 minutes. After washing, macrophages were incubated at 37°C for 60 minutes with a suspension of aged neutrophils (PMN), immunoglobulin G-opsonized erythrocytes (EIgG), zymosan, or medium alone (control). Non-ingested particles were washed away, and 0.5 ml of fresh serum-free medium was added. The medium was harvested at three hours, replaced, and then harvested again at 18 hours. Supernatants were centrifuged to remove cells and stored at –80°C prior to IL-8 ELISA. Results are presented as mean ± SEM of three independent experiments. For some conditions, one (†), or two (‡) results were below the lower limit of detection of the assay (10 pg/ml), or one result was above the maximum limit of detection (2000 pg/ml, ¥). * $P<0.05$, ** $P<0.01$ compared with control.

Macrophages fail to release IL-10 in response to phagocytosis of apoptotic neutrophils

The published literature contains two reports suggesting that release of certain cytokines may be stimulated by phagocytosis of apoptotic cells. Fadok and colleagues have suggested that exposure of human macrophages to apoptotic neutrophils induced production of transforming growth factor (TGF)-β, and that TGF-β in turn acted in an autocrine or paracrine manner to inhibit production of other cytokines such as TNF-α (Fadok et al., 1998). Moreover, production of IL-10 by human monocytes was reported to be stimulated following interaction with mixed peripheral blood mononuclear cells which had been induced to undergo apoptosis by irradiation (Voll et al., 1998). In response to this report, IL-10 was measured in the 18 hour supernatants from human monocyte-derived macrophages that had been exposed to CD44 antibody, apoptotic neutrophils, EIgG, zymosan, or LPS. Surprisingly, although neat samples were analysed by ELISA, there was virtually no detectable IL-10 under any condition except exposure to zymosan (figure 7). Certainly there was no evidence of stimulated production following phagocytosis of apoptotic neutrophils. It is extremely unlikely that all IL-10 production occurs in the first three hours following the phagocytic interaction, although it remains possible that analysis of the samples "neat" may have interfered with the sensitivity of the ELISA. The correspondence by Voll and colleagues is intriguing because the authors report stimulation of IL-10 following interaction of human monocytes with irradiated peripheral blood mononuclear cells, but they did not show any evidence that phagocytosis had actually occurred. In my hands peripheral blood monocytes are unable to phagocytose apoptotic neutrophils, this capability being acquired only after three days of maturation in vitro.

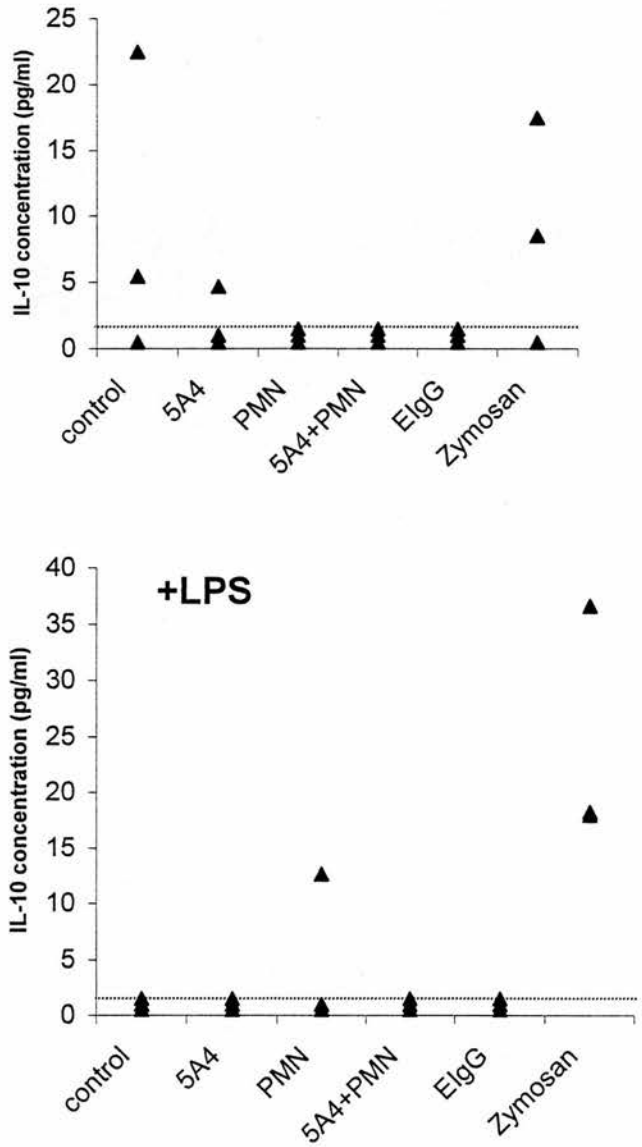


FIGURE 7

Release of IL-10 by macrophages in response to phagocytosis of apoptotic neutrophils

Human monocyte-derived macrophages were pre-incubated for four hours in medium alone (upper panel) or medium containing 50 ng/ml E. coli O18 lipopolysaccharide (lower panel). Cells were then washed three times prior to addition of CD44 antibody (5A4) or medium alone (control) for 30 minutes. After washing, macrophages were incubated at 37°C for 60 minutes with suspension of aged neutrophils (PMN), immunoglobulin G-opsonized erythrocytes (EIgG), zymosan, or medium alone (control). Non-ingested particles were washed away, and 0.5 ml of fresh serum-free medium was added. The medium was harvested at 18 hours. Supernatants were centrifuged to remove cells and stored at –80°C prior to IL-10 ELISA. Because so many of the samples contained concentrations of IL-10 below the lower limit of detection of the assay (1.5 pg/ml; plotted as a horizontal line), individual results from the three independent experiments have been plotted (closed triangles).

SUMMARY

CD44-augmented phagocytosis of apoptotic neutrophils was associated with low levels of measurable macrophage release of TNF-α and IL-8 in vitro, although not significantly lower than that following apoptotic cell ingestion in the absence of CD44 antibody even following macrophage stimulation with LPS. In contrast, ingestion of EIgG, which occurred at a lower rate than that of apoptotic neutrophils under basal conditions, resulted in significant macrophage release of pro-inflammatory cytokines. These observations are consistent with a model of macrophage phagocytosis of apoptotic neutrophils as a "silent" or anti-inflammatory process. Although earlier results suggested that CD44 ligation may result in recruitment of a distinct novel molecular mechanism for apoptotic neutrophil recognition (chapter 3), data presented here imply that it functions in a similar way to the basal recognition mechanism in terms of cytokine production.

Interestingly, ligation of CD44 on monocytes by antibody or macrophages by ligand (hyaluronan) has been reported to stimulate release of the inflammatory cytokines IL-1, M-CSF, or TNF-α (Webb et al., 1990; Gruber et al., 1992; Noble et al., 1993). Although macrophages tend to exhibit reduced secretory potential during their differentiation from monocytes, this study failed to demonstrate any effect of CD44 antibody on release of TNF-α or IL-8. The effect of hyaluronan on macrophage cytokine release was not tested in the present study, but the data suggest that CD44 ligation by antibody and hyaluronan induce different functional responses. It should be remembered that many other physiological ligands for CD44 have been nominated (Lesley et al., 1993), and that different sized hyaluronan fragments may themselves exert distinct effects on mononuclear phagocyte function (Noble et al., 1993; McKee et al., 1996).

Analysis of transcription factor activation, cytokine synthesis by PCR or northern blotting, and intracellular localisation of cytokine stores by immunocytochemistry could give valuable additional information about the macrophage response to CD44 ligation and phagocytosis of apoptotic neutrophils. It would be also be interesting in further studies to also assess the effect of CD44-augmented phagocytosis on macrophage release of enzymes, prostanoids, different cytokines, and other inflammatory mediators.

INTRACELLULAR SIGNALLING EVENTS FOLLOWING MACROPHAGE CD44 LIGATION

INTRODUCTION

Macrophages at an inflammatory site may be exposed to a variety of external signals that control and coordinate clearance of apoptotic cells, and a key role for ligation of macrophage CD44 in this process is supported by results of experiments presented in this thesis (figure 8).

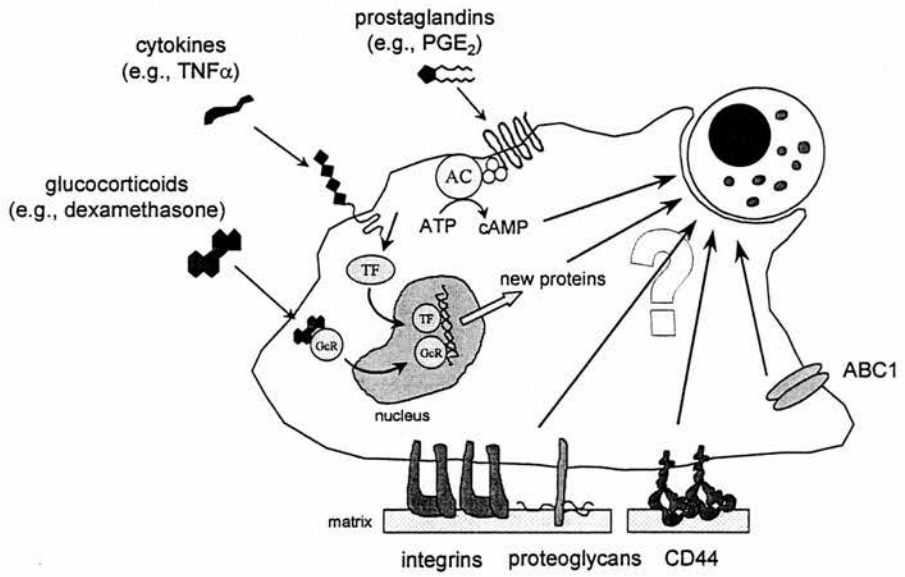


FIGURE 8

Surface receptors and intracellular signalling pathways that may regulate macrophage phagocytosis of apoptotic cells.

Macrophage recognition of apoptotic cells may be controlled by cytokines (Ren and Savill, 1995), steroid hormones (Liu et al., 1999), prostaglandins (Rossi et al., 1998), and adhesion to matrix molecules such as fibronectin (McCutcheon et al., 1998).

(AC, adenylate cyclase; ABC1, ATP binding cassette transporter 1; GcR, glucocorticoid receptor; TF, transcription factor)

It has been shown that CD44 antibodies augmented macrophage phagocytosis of apoptotic neutrophils following incubation for 20 minutes prior to a 30 minute in vitro phagocytosis assay. Further experiments demonstrated that pre-incubation with CD44 antibody for as little as five minutes was sufficient to significantly enhance apoptotic neutrophil uptake (S. Hart and I. Dransfield, unpublished observations). The rapidity of effect of CD44 antibodies, together with evidence that CD44 was not directly involved in tethering of the phagocyte to the apoptotic cell (chapter 3), suggests that ligation of macrophage CD44 by antibodies may trigger intracellular signalling pathways that lead to increased phagocytosis of apoptotic neutrophils. CD44 may associate with the Src family tyrosine kinases Lck and Fyn in lymphocytes (Ilangumaran et al., 1998), and CD44 ligation has been shown to rapidly induce tyrosine phosphorylation of $p56^{lck}$ (Taher et al., 1996) and to elevate intracellular cAMP concentrations (Rothman et al., 1993). The latter effect does not explain the effect of CD44 antibody on phagocytosis because treatment of macrophages with dibutyryl cAMP (a membrane-permeable analogue of cAMP) inhibited rather than

stimulated phagocytosis of apoptotic neutrophils (Rossi et al., 1998). Alternatively, binding of monoclonal antibodies may modify associations between CD44 and cytoskeletal elements, including members of the ezrin-radixin-moiesin (ERM) family, or other membrane molecules that act indirectly to influence macrophage phagocytic capability (Camp et al., 1991; Tsukita et al., 1994; Isacke, 1994).

Intact cytoskeletal microtubules and microfilaments are clearly required for macrophage phagocytosis of apoptotic cells since ingestion was substantially inhibited when these elements were inhibited (chapter 4). However, stabilisation of microtubules by elevation of intracellular cGMP had no effect on phagocytosis of apoptotic neutrophils (Rossi et al., 1998), which contrasts with the particular importance of microtubular integrity for CR1- and CR3-mediated particle ingestion (Newman et al., 1991). This disparity illustrates again the differential involvement of cytoskeletal components in apoptotic cell clearance compared with other phagocytic pathways. Recent studies of C. elegans mutants that display defective phagocytic removal of cellular corpses have characterised a number of genes involved in this process that may give important clues about the nature of mammalian apoptotic cell recognition pathways. For example, the ced-6 gene product contains sequences similar to mammalian phosphotyrosine binding domains (Liu and Hengartner, 1998), and the protein encoded for by ced-5 shows homology to the human CRK-binding protein DOCK-180 that has been implicated in cytoskeletal function and extension of cell surfaces (Wu and Horvitz, 1998). Interestingly, preliminary observations had suggested that ligation of CD44 by antibodies resulted to subtle morphological changes and reorganisation of the actin cytoskeleton as determined by staining with rhodamine-phalloidin (I. Dransfield, unpublished observations). However, because of the inherent heterogeneity of human monocyte-derived macrophages this observation has proved very difficult to quantify. As an alternative approach it was decided to analyse different components of the cytoskeleton by immunoprecipitation and Western blotting. Candidate molecules that were analysed included the cytoskeletal linker proteins of the ERM family, Lck, and other cytoskeleton-associated proteins such as paxillin, that are known to be involved in cytoskeletal reorganisation and phagosome formation (Greenberg et al., 1994; Bellis et al., 1997).

RESULTS AND DISCUSSION

Western blotting of macrophage lysates with phosphotyrosine antibody

Because CD44 has been reported to be associated with tyrosine kinases in lymphocytes (Ilangumaran et al., 1998; Taher et al., 1996), the pattern of tyrosine-phosphorylated proteins in human monocyte-derived macrophage following treatment with CD44 antibody 5A4 was analysed. In an initial series of experiments, lysates of control and 5A4-treated macrophages were subjected to Western blotting with an anti-phosphotyrosine antibody, RC20. This simple approach was thought to represent a reasonable initial screening method to detect alterations in tyrosine phosphorylated proteins, but it is insensitive since only small quantities of the proteins of interest are present compared with the background "noise". The results of a typical experiment are presented in figure 9. Careful scrutiny fails to reveal any convincing differences in the pattern of tyrosine-phosphorylated proteins between untreated and CD44 antibody-treated macrophages.

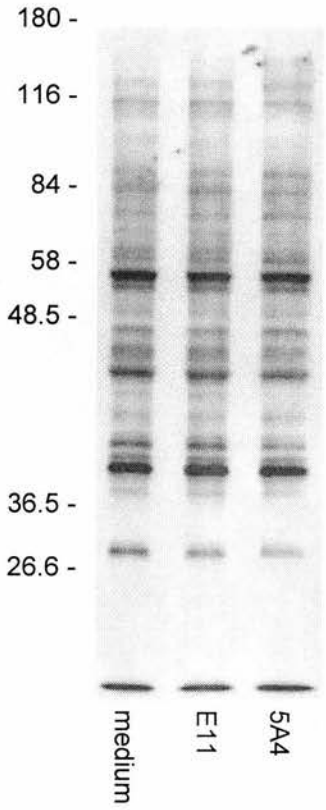


FIGURE 9
Macrophage tyrosine phosphorylated proteins following CD44 ligation with antibody 5A4
Human monocyte-derived macrophages were incubated with medium alone, E11 (anti-CR1, control), or 5A4 (anti-CD44) for 30 minutes. Cells were lysed and the lysates subjected to SDS-PAGE before Western blotting with HRP-conjugated RC20 (anti-phosphotyrosine). Bands were visualised with enhanced chemiluminescence.

To increase the sensitivity of detection of altered tyrosine phosphorylation, candidate intracellular proteins of interest were immunoprecipitated prior to Western blotting with a phosphotyrosine antibody. In addition to tyrosine kinases such as Lck and Fyn, CD44 has been reported to associate with cytoskeletal components, especially members of the ERM family of cytoskeletal linker proteins (Tsukita et al., 1994). Commercially available antibodies were used to immunoprecipitate tyrosine phosphorylated proteins (antibody PY20), ezrin, paxillin, and Lck from macrophage lysates. Immunoprecipitates were subjected to SDS-PAGE, blotted onto nitrocellulose, and then probed with an phosphotyrosine antibody (RC20) linked to HRP which permitted visualisation with enhanced chemiluminescence (figure 10).

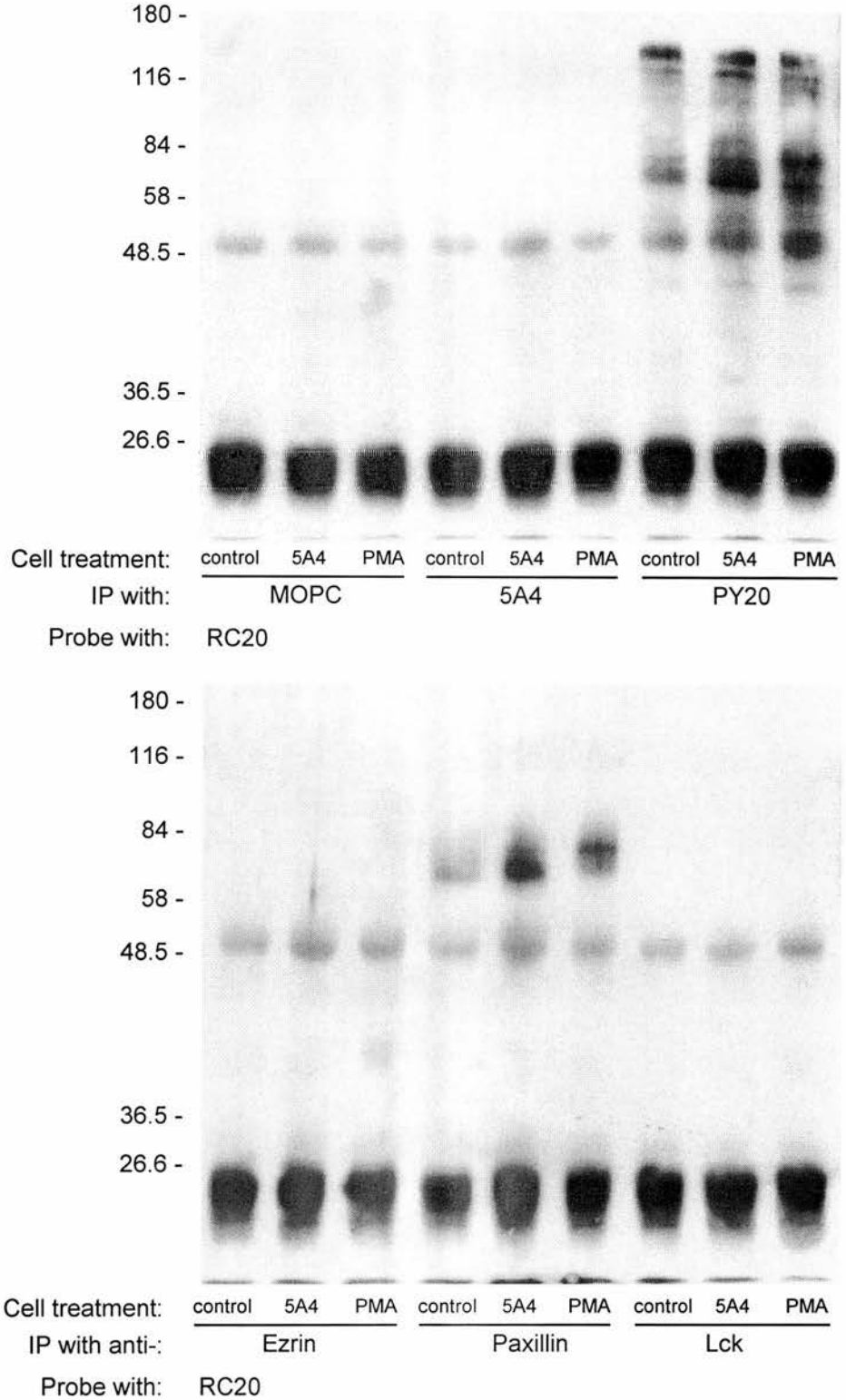


FIGURE 10

Macrophage tyrosine phosphorylation of proteins following CD44 ligation with antibody5A4

Human monocyte-derived macrophages were incubated with PBS containing W6/32 (anti-MHC I control 1:5 supernatant), 5A4 (anti-CD44 1:5 supernatant), or PMA (160 nM) for 40 minutes. The cells were then lysed with Tris-buffered saline (pH 7.5) containing 1% NP-40 and protease inhibitors. The lysates were incubated with monoclonal antibodies MOPC (non-binding control), 5A4 (anti-CD44), PY20 (anti-phosphotyrosine), anti-ezrin, anti-paxillin, or anti-Lck, followed by anti-mouse Ig-agarose. The pellets were washed and boiled in reducing Laemmli SDS sample buffer. Immunoprecipitates were run on a 10% polyacrylamide gel and electroblotted onto nitrocellulose. Tyrosine phosphorylated proteins were visualised by incubation with RC20 (anti-phosphotyrosine)-HRP and development using enhanced chemiluminescence. Constant bands of approximately 50 kD and 25 kD represent antibody heavy and light chains respectively.

Examination of the pattern of PY20 (anti-phosphotyrosine) immunoprecipitates suggested that macrophage CD44 ligation with 5A4 resulted in increased tyrosine phosphorylation of a protein or proteins with a MW 60-70 kD. Interestingly, treatment with PMA similarly increased tyrosine phosphorylation of proteins of similar MW, but was also associated with the presence of a more slowly migrating tyrosine-phosphorylated band on the gel. This suggests that PMA may induce a shift to a higher molecular weight isoform, perhaps related to phosphorylation of serine and threonine residues. This characteristic pattern of bands seemed to be identical to those present in the anti-paxillin immunoprecipitates, suggesting that paxillin or a protein that co-immunoprecipitates with it underwent increased tyrosine phosphorylation following CD44 ligation in macrophages. Paxillin has been reported to have a MW of 68 kD (Mazaki et al., 1997), suggesting that the tyrosine phosphorylated bands represented paxillin itself.

No tyrosine phosphorylated bands were observed in the lanes representing anti-ezrin or anti-Lck immunoprecipitates. Possible explanations for this negative result were that these proteins were not present in human monocyte-derived macrophages; the antibodies used failed to extract their respective antigens; or that neither of these proteins was tyrosine phosphorylated. Western blotting of cell lysates was performed to determine whether these molecules are present in human monocyte-derived macrophages (figure 11).

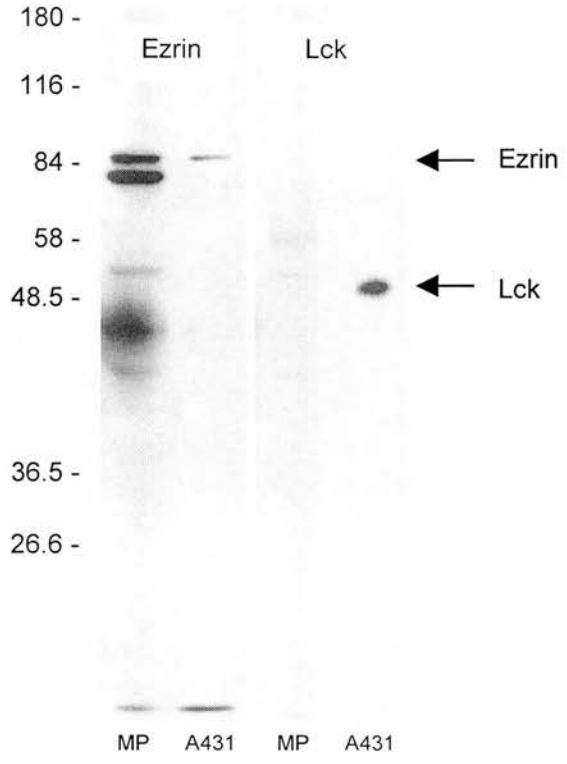


FIGURE 11
Ezrin but not Lck is present in human monocyte-derived macrophages
Lysates of macrophages or EGF-stimulated A431 cells (positive control) were subjected to SDS-PAGE and blotted onto nitrocellulose membranes. The membranes were then probed with ezrin or Lck antibodies followed by anti-mouse Ig-HRP and visualised by enhanced chemiluminescence.

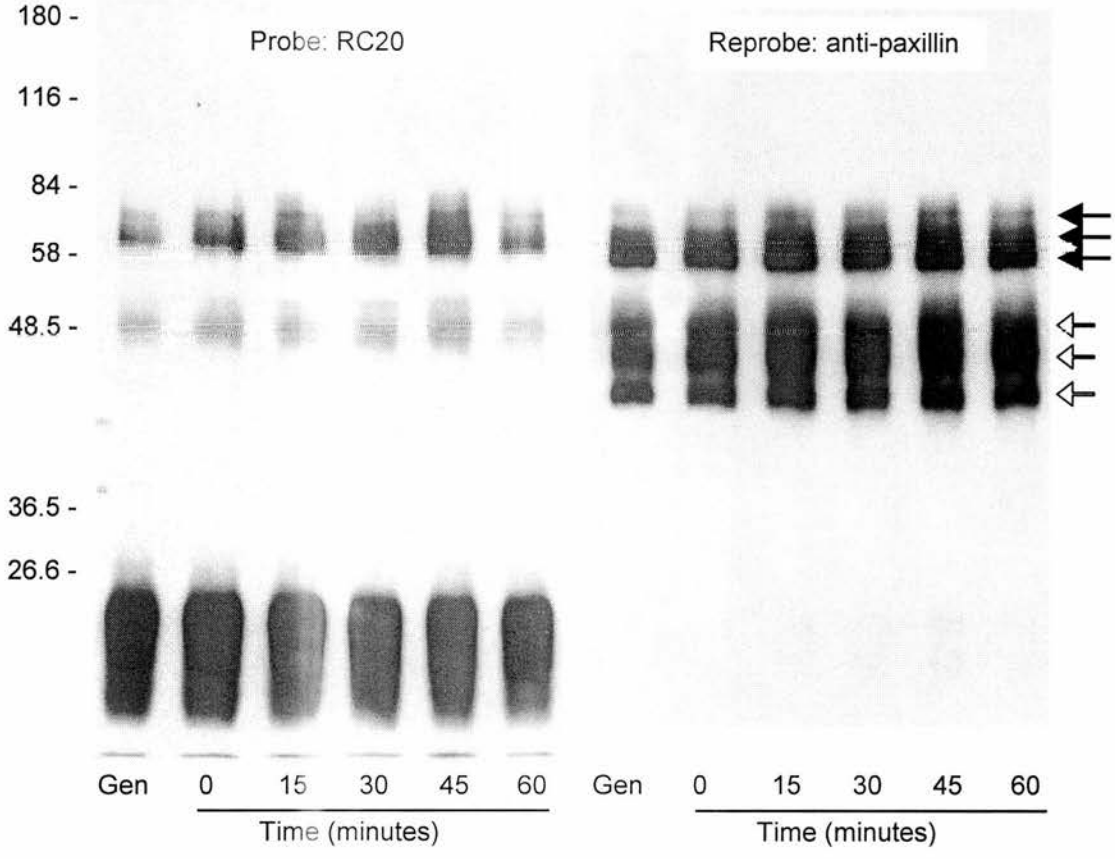
Western blotting demonstrated that ezrin but not Lck immunoreactivity was present in human monocyte-derived macrophages. Further experiments showed that although this ezrin antibody performed well in Western blotting, it failed to immunoprecipitate ezrin from macrophage lysates. This observation meant that using this particular antibody it was not possible to determine whether ezrin underwent tyrosine phosphorylation in response to macrophage CD44 ligation.

Tyrosine phosphorylation of paxillin

Paxillin is a cytoskeletal-associated protein that is recruited to focal adhesions. The recruitment and activity of paxillin at focal adhesions is controlled by phosphorylation of tyrosine residues (Bellis et al., 1995). It has been established that ligation of integrins stimulates tyrosine phosphorylation of paxillin in a variety of cell types (Fuortes et al., 1994; Graham et al., 1994; De Nichilo and Yamada, 1996; Bellis et al., 1997), but interestingly, Fc-receptor mediated phagocytosis has been reported to be associated with tyrosine phosphorylation of paxillin in macrophages (Greenberg et al., 1994). To further investigate the tyrosine phosphorylation of paxillin in response to CD44 antibody a series of time course experiments were performed. In three independent experiments there appeared to be time-dependent tyrosine phosphorylation of paxillin in response to binding of CD44 antibody 5A4 (figure 12). Furthermore, a number of paxillin isoforms appeared to be present in macrophages. At least three separate bands could be identified in the 60-80 kD range. Interestingly, the lowest MW isoform of this group was the most prevalent but appeared not to be tyrosine phosphorylated. The increase in tyrosine phosphorylation of

paxillin observed with CD44 antibody 5A4 (or with PMA) may be associated with an increase in the prevalence of the higher MW isoforms. In addition, a parallel series of lower MW isoforms (40-50 kD) were also present. These may represent proteolytic fragments of the higher MW paxillin, and they reacted relatively weakly with phosphotyrosine antibody RC20. It was notable that performing this experiment under stringent conditions designed to minimise proteolysis had no effect on the presence of these lower MW bands.

FIGURE 12
Time course of tyrosine phosphorylation of macrophage paxillin following CD44 ligation



Human monocyte-derived macrophages were washed and incubated with genestein or antibody 5A4 for 0-60 minutes. The cells were washed and then lysed with Tris-buffered saline (pH 7.5) containing 1% NP-40 and protease inhibitors. Paxillin was immunoprecipitated from the macrophage lysates, subjected to SDS-PAGE, and electroblotted onto nitrocellulose. Tyrosine phosphorylation of paxillin IP products was detected by incubation with RC20-HRP and enhanced chemiluminescence (left panel). The blot was then stripped and reprobed with anti-paxillin and anti-mouse Ig-HRP to ensure that comparable amounts of paxillin had been extracted from each sample (right panel).
Note that macrophage paxillin seems to comprise a series of isoforms close to the reported MW of 68 kD (closed arrows), and a series of lower MW isoforms which could represent proteolytic fragments (open arrows). The principal high MW isoform is apparently not tyrosine phosphorylated (lower closed arrow).

Whether tyrosine phosphorylation of paxillin is causally associated with augmented phagocytosis of apoptotic neutrophils following CD44 ligation remains to be determined. It would be interesting to analyse the localisation of paxillin within macrophages by immunocytochemistry, and to determine whether it appears to co-localise with other molecules such as CD44, ERM proteins, of focal adhesion

kinase following treatment with CD44 antibodies. Experiments designed to co-immunoprecipitate these proteins with CD44 antibodies have been disappointing, although it may be possible to do so using fusion proteins comprising the cytoplasmic tail of CD44 linked to Ig-Fc. Phosphorylation on serine or threonine residues may also be an important intracellular signalling pathway. Further experiments using phosphate labelling could give useful information about other phosphorylation events that may occur following macrophage CD44 ligation. Preliminary experiments using new commercially available phosphoserine/phosphothreonine antibodies have shown that these reagents exhibit considerably more non-specific binding than the phosphotyrosine antibodies used in the present study.

SUMMARY

CD44 appears not to be directly involved in phagocytosis of apoptotic cells, and the observation that CD44 ligation rapidly augments phagocytosis of apoptotic neutrophils suggested that intracellular signal transduction pathways may be involved. Although CD44 has been reported to be associated with tyrosine kinases in lymphocytes (Ilangumaran et al., 1998; Taher et al., 1996), the pattern of tyrosine-phosphorylated proteins in human monocyte-derived macrophages was unchanged following treatment with CD44 antibody 5A4. However, when a more sensitive approach was used involving immunoprecipitation of tyrosine phosphorylated proteins, ezrin, paxillin, and Lck from macrophage lysates prior to Western blotting with phosphotyrosine antibody, more convincing results were obtained. These experiments demonstrated that macrophage CD44 ligation with 5A4 resulted in increased tyrosine phosphorylation of a protein or proteins with a MW 60-70 kD. This characteristic pattern of bands seemed to be identical to those present in the anti-paxillin immunoprecipitates, suggesting that paxillin or a protein that co-immunoprecipitates with it underwent increased tyrosine phosphorylation following CD44 ligation in macrophages. Paxillin has been reported to have a MW of 68 kD, suggesting that the tyrosine phosphorylated bands represented paxillin itself. Western blotting demonstrated that ezrin but not Lck was present in human monocyte-derived macrophages. Further experiments demonstrated a time-dependent increase in tyrosine phosphorylation of paxillin following CD44 ligation.

However, the importance of tyrosine phosphorylation of paxillin following CD44 ligation in relationship to augmented phagocytosis of apoptotic neutrophils is unclear. Paxillin is phosphorylated in response to a variety of signals in macrophages, including activation of integrins (Bellis et al., 1997). Antibodies to a variety of integrin subunits have no demonstrable effect on phagocytosis of apoptotic neutrophils (I. Dransfield, personal communication), although adhesion of macrophages to fibronectin augments phagocytosis by a mechanism that depends at least partially on activation of β1 integrins (McCutcheon et al., 1998). Furthermore, tyrosine phosphorylation of paxillin has been reported to be associated with phagocytosis of IgG-opsonized erythrocytes by mouse macrophages (Greenberg et al., 1994), which can be completely inhibited with broad spectrum tyrosine kinase inhibitors such as genestein (Greenberg et al., 1993). Tyrosine phosphorylation of paxillin therefore seems to be a relatively non-specific feature of macrophage activation, and is therefore not likely to be causally related to the observed specific effect of CD44 antibody on phagocytosis of apoptotic neutrophils.

Moreover, macrophages appeared to contain a number of paxillin isoforms, some of which may have represented proteolytic fragments. Similar isoforms have been identified in previous studies (Mazaki et

al., 1997), although their significance is not understood. Further experiments using genetically modified ("knockout") animals or antisense technology could be used to further elucidate the role of paxillin in apoptotic cell clearance by macrophages.

CHAPTER 5
MOLECULAR CHARACTERISATION OF THE APOPTOTIC NEUTROPHIL SURFACE

INTRODUCTION

By down-regulating neutrophil functions and triggering their anti-inflammatory clearance by phagocytes, apoptosis provides a mechanism for the safe disposal of inflammatory cells. It is likely that significant changes in the protein, lipid, and carbohydrate composition of the apoptotic neutrophil plasma membrane play an important role in the "functional isolation" and subsequent phagocytic clearance of these potentially destructive dying cells. For example, it has been shown that neutrophil apoptosis is associated with reduced expression and/or function of cellular adhesion molecules, so diminishing the activation responses that normally result from interaction with the extracellular matrix and neighbouring cells (Dransfield et al., 1995).

The molecular alterations on the surface of the apoptotic neutrophil that are responsible for phagocyte recognition have yet to be characterised. Much attention has been focussed on the anionic phospholipid phosphatidylserine (PS) (Verhoven et al., 1995), which is relocated from the inner to the outer surface of the plasma membrane during apoptosis (Homburg et al., 1995). PS exposure has been implicated in the recognition of apoptotic leukocytes by mouse inflammatory macrophages in vitro (Fadok et al., 1992b), but PS may not be involved in apoptotic cell recognition by other phagocytes, including human macrophages (Fadok et al., 1992a; Hart et al., 1996). Considering the diversity of phagocyte surface molecules that have been proposed to be involved in the recognition of apoptotic cells (including lectins, the integrin $\alpha_V\beta_3$ (CD51/61), CD36, a PS receptor, a scavenger receptor, ABC transporters, and CD14 (Savill et al., 1993; Hart et al., 1996; Devitt et al., 1998)), it is likely that additional undefined molecular changes on the apoptotic cell surface act as phagocyte recognition signals under different circumstances.

It has been demonstrated that apoptotic and non-apoptotic neutrophils in a mixed population of aged cells can be defined on the basis of differential expression of FcγRIII (CD16) (Dransfield et al., 1994). This observation has been exploited to characterise changes in the surface expression of proteins associated with neutrophil apoptosis by using dual colour flow cytometric analysis with a panel of monoclonal antibodies (S.P.Hart, J.A.Ross, C.Haslett, I.Dransfield, submitted). Using this approach, a monoclonal antibody, Bob93, has been identified that binds specifically to apoptotic ($CD16^{low}$) neutrophils (J.A. Ross and I.Dransfield, unpublished observations). This chapter describes further characterisation of the Bob93 antigen together with analysis of the apoptotic neutrophil carbohydrate profile using dual colour flow cytometric analysis with a panel of labelled lectins. These studies define molecular alterations that may play important roles in the down-regulation of function and the recognition of apoptotic neutrophils by phagocytes.

RESULTS AND DISCUSSION

Neutrophil surface carbohydrates

It has been proposed that desialated sugars expressed on apoptotic neonatal rat liver cells play a role in their recognition by phagocyte asialoglycoprotein receptors (Dini et al., 1992). In contrast, examination of the surface of apoptotic mouse thymocytes failed to show any change in sugar expression (Morris et al., 1984). Interestingly, brief treatment of apoptotic neutrophils with Vibrio cholerae neuraminidase (which cleaves terminal sialic acid residues) significantly augmented their phagocytosis by human monocyte-derived macrophages in vitro (chapter 4), whereas treatment of the apoptotic cells with broad spectrum proteases had little effect on recognition. These findings are consistent with the proposal that altered exposure of carbohydrate moieties on apoptotic neutrophils may provide a signal for phagocytic clearance by macrophages.

Examination of the patterns of surface carbohydrate expression on cultured human neutrophils using dual colour flow cytometric analysis with antibodies to the sialated surface glycan sialyl-Lewis^x (sCD15) revealed that expression of sCD15 was reduced by approximately 80% on apoptotic neutrophils (Dransfield et al., 1995). However, decreased expression of sCD15 paralleled that observed for non-sialated CD15 (Lewis^x; lactosyl fucosyl pentaose; S.P.Hart, J.A.Ross, C.Haslett, I.Dransfield, submitted). This observation supports the suggestion that human neutrophil apoptosis is not associated with specific loss of sialated sugars. However, the CD15 carbohydrate is present on a number of different neutrophil proteins including CD18 and CD66, but expression of these receptors is largely maintained during neutrophil apoptosis (Dransfield et al., 1995)(S.P.Hart, J.A.Ross, C.Haslett, I.Dransfield, submitted), raising the possibility that specific deglycosylation events may accompany apoptosis.

Lectin binding studies

Because of the limited availability of antibodies that bind to defined carbohydrate structures, a panel of labelled lectins was utilised to extend the analysis of carbohydrates on the surface of apoptotic neutrophils. Dual colour flow cytometric analysis was performed with CD16 antibodies in conjunction with eight different labelled lectins, each with relative specificity for particular carbohydrate structures. Binding of each lectin to the surface of apoptotic neutrophils was moderately reduced compared with non-apoptotic cells (figure 1). In contrast, neuraminidase-treated neutrophils exhibited a 95% reduction in MAL2 binding and a 1100% increase in PNA binding compared with untreated cells, confirming loss of sialic acid and exposure of galactose residues (figure 2).

A further simple experiment was performed to show that the reduction of lectin binding to CD16^{low} apoptotic neutrophils was not a result of reduced binding of glycosylated CD16 antibody. Freshly isolated neutrophils were split and half of the cells were incubated with CD16 antibody, washed, and then mixed with the unlabelled cells. There was no difference in lectin binding to the CD16-labelled neutrophils compared with the unlabelled cells.

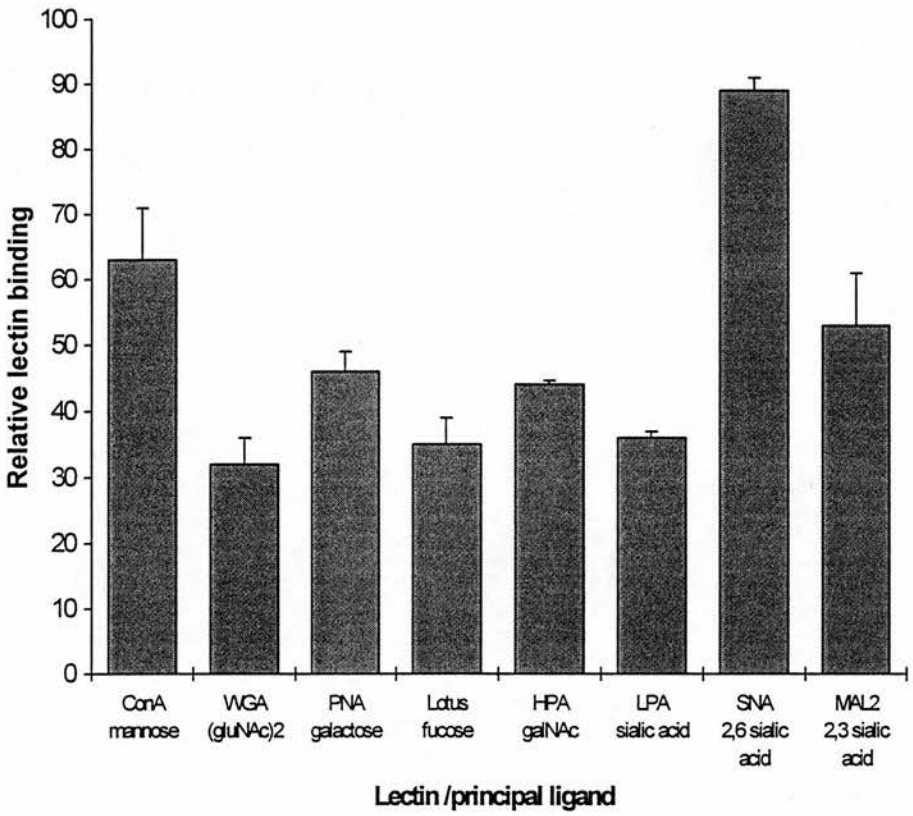


FIGURE 1
Neutrophil apoptosis is associated with generalised loss of surface sugars.

Aged human peripheral blood neutrophils (approximately 50% apoptotic) were incubated on ice with CD16 antibody 3G8, washed, and then incubated with $F(ab')_2$ goat anti-mouse PE together with the FITC-labelled lectin (except for MAL2 where a biotinylated lectin was used followed by streptavidin-FITC). The cells were washed and dual colour flow cytometric analysis was performed following appropriate colour compensation. The binding of lectins to apoptotic ($CD16^{low}$) neutrophils is expressed as a percentage of the binding to non-apoptotic ($CD16^{high}$) neutrophils. Values are mean ± SEM of three independent experiments. $P<0.05$ for all lectins. For abbreviations see chapter 2 (methods).

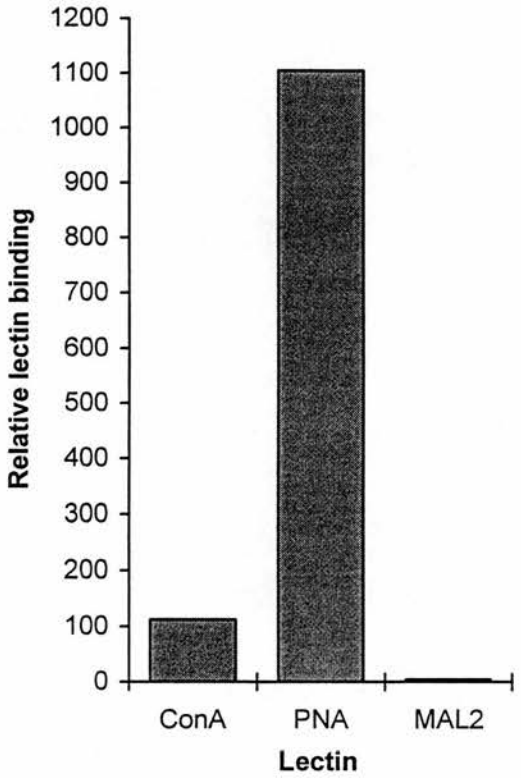


FIGURE 2
Effect of neuraminidase treatment on lectin binding

Freshly isolated peripheral blood neutrophils were treated with 0.1 U/ml Vibrio cholerae neuraminidase prior to assessment of lectin binding. Results from one representative experiment are shown.

Together, these data suggest that desialation of surface carbohydrates associated with neutrophil apoptosis is non-specific and relatively subtle. Loss of carbohydrate is unlikely to represent the only determinant of recognition and phagocytosis of apoptotic neutrophils, although it remains possible that the degree of glycosylation of specific molecules may influence the phagocytic response. Certain heavily glycosylated neutrophil receptors such as CD43, CD44, and CD50 are down-regulated during neutrophil apoptosis. It is tempting to speculate that loss of these receptors may partly account for the reduction in binding of sialic acid-specific lectins in this study. Recent data suggests that CD43 is a negative regulator of lymphocyte adhesion processes (Stockton et al., 1998), raising the possibility that loss of CD43 or other heavily sialated receptors may facilitate adhesive interactions between apoptotic neutrophils and macrophages that lead to phagocytosis.

Bob93: a monoclonal antibody that binds to mononuclear phagocytes and apoptotic neutrophils

A panel of hybridomas had been generated by immunizing mice with human THP-1 cells (a primitive monocytoid cell line). By screening the hybridoma supernatants using dual colour indirect immunofluorescence and flow cytometric analysis a monoclonal antibody, called Bob93, was identified that bound specifically to apoptotic neutrophils. It was found that freshly isolated neutrophils bound Bob93 very weakly. However, when neutrophils underwent apoptosis during culture in vitro there was time-dependent increase in Bob93 binding that closely mirrored loss of CD16, a well-established marker for neutrophil apoptosis (Dransfield et al., 1994; I.Dransfield, unpublished observations).

In an extension of these experiments to other live human cells, the Bob93 antigen was found to be expressed on the surface of only mononuclear phagocytes (monocytes and monocyte-derived macrophages), but was undetectable on erythrocytes, lymphocytes, and eosinophils (figure 3).

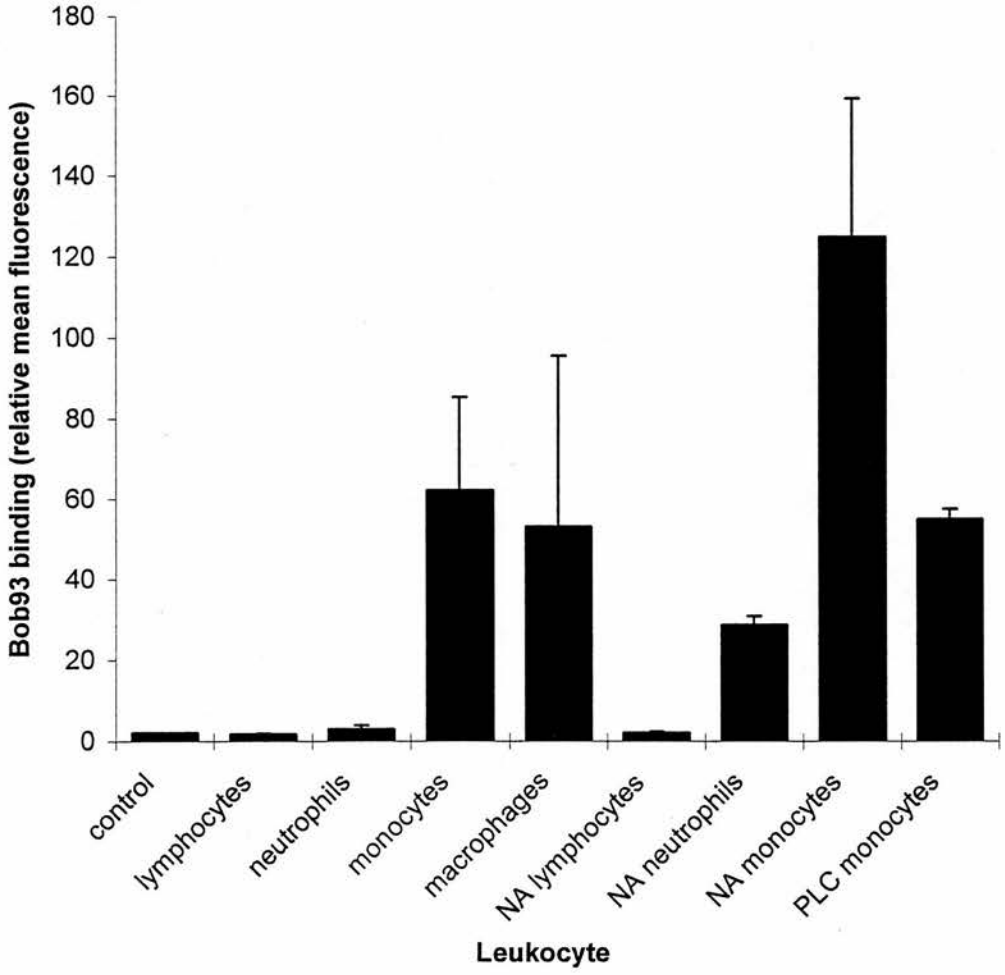


FIGURE 3
Binding of Bob93 to leukocytes.
Bob93 (in hybridoma supernatant) binding to human leukocytes was assessed by indirect immunofluorescence and flow cytometric analysis. Some cells were pre-treated with Vibrio cholerae neuraminidase (0.1 U/ml for 15 min at 37°C) ("NA") or PI-PLC (1 U/ml for 30 min at 37°C) ("PLC"). Values are mean ± SEM of at least three independent experiments.

Binding of Bob93 to aged neutrophils exhibited a bimodal pattern, representing apoptotic (Bob93^{high}) and non-apoptotic (Bob93^{low}) neutrophils within the cell population (figure 4). In contrast, Bob93 binding to lymphocytes and eosinophils that had been induced to undergo apoptosis during in vitro culture exhibited only a single Bob93^{low} population. Although this is an insensitive method of distinguishing binding to apoptotic and non-apoptotic cells within a mixed population, these observations suggest that Bob93 may not bind to apoptotic lymphocytes or eosinophils, and that the Bob93 antigen is specifically expressed on mononuclear phagocytes and apoptotic neutrophils.

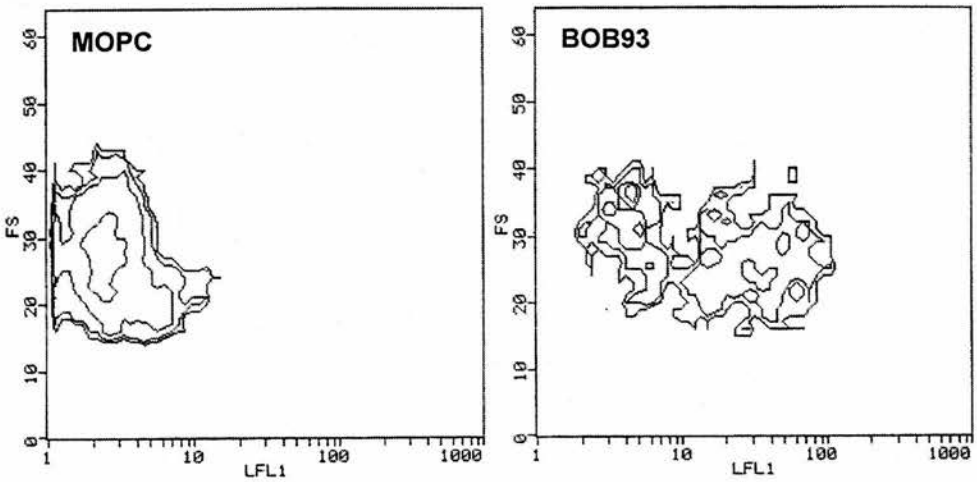


FIGURE 4
Flow cytometry profiles of Bob93 binding to aged neutrophils
Human peripheral blood neutrophils were aged for 20 hours at 37°C in vitro during which time a proportion of the cells underwent apoptosis. Binding of antibody Bob93 (or control antibody MOPC) was assessed by indirect immunofluorescence and flow cytometry. Bob93 bound to a population of aged neutrophils which had a slightly lower average forward scatter than Bob93^{low} cells. Dual labelling with Bob93 and CD16 antibody demonstrated that Bob93^{high} cells represented CD16^{low} apoptotic neutrophils (I. Dransfield, unpublished observations).

To characterise the nature of the interaction between Bob93 and its cell surface antigen, the effect of enzyme treatment of the cell surface was examined. Occult Bob93 binding sites on freshly isolated neutrophils were unmasked following treatment of the cells with Vibrio cholerae neuraminidase (figure 3). Similarly, Bob93 binding to monocytes was increased twofold following treatment with neuraminidase. In contrast with monocytes and neutrophils, peripheral blood lymphocytes and eosinophils did not exhibit any Bob93 binding even following desialation with neuraminidase. Although neuraminidase treatment does not mimic changes that occur during neutrophil apoptosis (see lectin binding section above), these observations support the suggestion that unlike neutrophils, lymphocytes and eosinophils do not have the potential to expose the Bob93 antigen during apoptosis.

Bob93 binding to monocytes could be abolished by brief treatment of the cells with trypsin (figure 5). Addition of protease inhibitors completely reversed the effect of trypsin and actually increased Bob93 binding compared with control levels, suggesting that this trypsin preparation (prepared from porcine pancreas) contained some neuraminidase-like activity. Treatment of monocytes with phosphatidylinositol-specific phospholipase C (PI-PLC), which cleaves GPI-linked molecules from the cell surface, had no effect on Bob93 binding (figure 3).

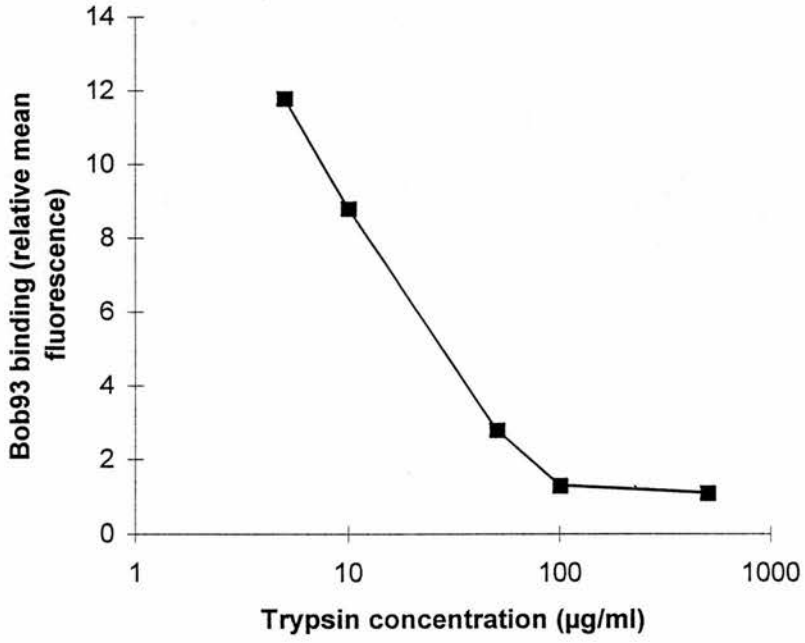
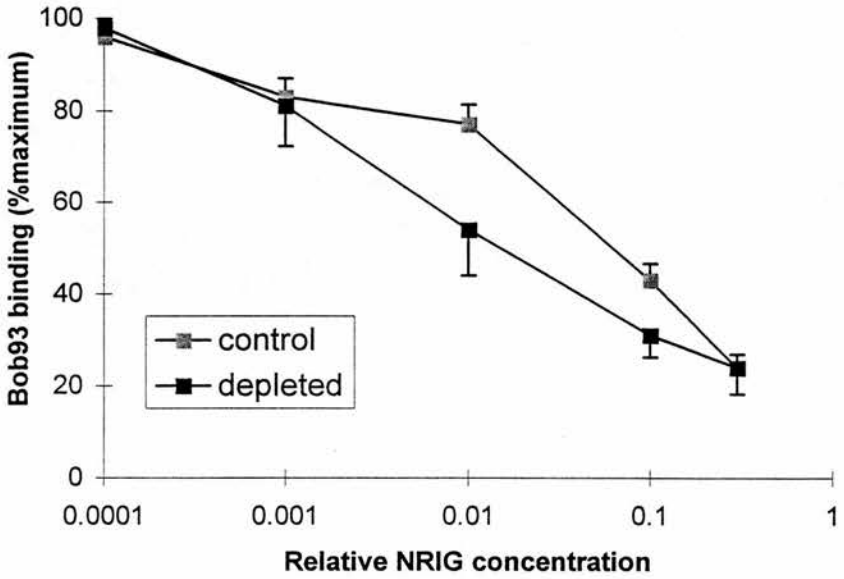


FIGURE 5.
Treatment of monocytes with trypsin induces a concentration-dependent loss of Bob93 binding
Human peripheral blood mononuclear cells were incubated with trypsin type IX (from porcine pancreas) for 30 minutes at 37°C in Iscove's DMEM. The cells were washed twice and then Bob93 binding was assessed by indirect immunofluorescence and flow cytometry as previously described. Data are mean log fluorescence values from one experiment.

Inhibition of Bob93 binding by rabbit serum

Non-immune rabbit immunoglobulin (NRIG), which is prepared from rabbit serum by salting out and ion exchange chromatography, has been traditionally used to block binding of antibodies to Fc receptors on the leukocyte surface. By chance it was discovered that binding of Bob93 was inhibited by co-incubation of monocytes or apoptotic neutrophils with NRIG in a concentration-dependent manner (figure 6), and further investigation of this phenomenon was carried out to provide information about the nature of the Bob93 antigen.



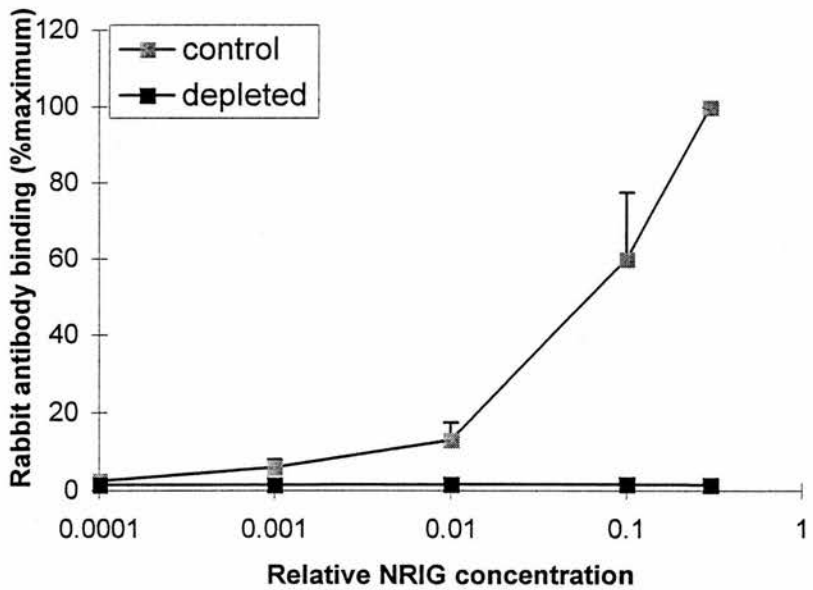


FIGURE 6.
Inhibitory effect of non-immune rabbit immunoglobulin on Bob93 binding.
NRIG was depleted of IgG by incubation with protein G-sepharose, or sham depleted with glycine-sepharose (control). Dilutions (where 1.0 represents undiluted NRIG with a protein concentration of 20 mg/ml) of control and depleted NRIG were incubated with monocytes for 10 min on ice prior to assessment of Bob93 binding by indirect immunofluorescence and flow cytometric analysis. IgG depletion was confirmed by elimination of binding to monocytes as assessed using a donkey anti-rabbit FITC second layer antibody (lower panel). IgG-depleted NRIG inhibited Bob93 binding to monocytes as effectively as sham depleted NRIG (upper panel).

Inhibition of Bob93 binding by NRIG suggested that Bob93 may recognise leukocyte receptors for the constant region of IgG (FcγR). The three classes of FcγR that have been characterised to date are differentially expressed by leukocytes. The high affinity Fc receptor, FcγRI (CD64), is expressed by monocytes, but is absent on neutrophils. The low affinity Fc receptors FcγRII (CD32) and FcγRIII (CD16) are present on both cell types, but both are down-regulated on apoptotic neutrophils (Dransfield et al., 1994; Dransfield et al., 1995). These patterns of expression suggested that IgG binding to Fc receptors was unlikely to be responsible for inhibition of Bob93 binding. Furthermore, NRIG that had been depleted of IgG with protein G-sepharose retained its inhibitory effect on Bob93 binding (figure 6). Moreover, pre-incubation of monocytes with "non-binding" antibodies of the IgG1 or IgG2a subclass had no significant effect on Bob93 binding to monocytes (table I), and saturating concentrations of the FcγRII antibody IV3 had little effect (table II). Together, these data strongly suggest that a component of the NRIG preparation other than IgG was responsible for inhibition of Bob93 binding.

Antibody	Cell binding (mean ± SEM; n=2)
Control (MOPC)	2.1
Bob93	31.4 ± 8.5
NC1 (IgM) + Bob93	33.2 ± 5.0
NC2 (IgG2a) + Bob93	33.6 ± 6.3
NC3 (IgG1) + Bob93	64.6 ± 3.1

TABLE I
Effect of different murine Ig subclasses on Bob93 binding to human monocytes.
Cells were incubated on ice for 15 minutes with NC1, NC2, or NC3 (10 µl; 200 µg/ml) before addition of Bob93 supernatant (40 µl) for a further 30 minutes. The cells were washed, incubated with 1:40 FITC labelled Fab'2 goat anti-mouse Ig for 30 minutes, washed again and analysed with an EPICS Profile II flow cytometer. The mean log fluorescence was recorded for each sample. The murine antibodies NC1, NC2, and NC3 had been submitted to the Fifth International Workshop on Leukocyte Typing and were described as non-binding. However, it is apparent from the data presented in this table that NC3 did bind to the monocyte surface, and this was confirmed in a separate experiment using NC3 alone.

Antibody	Bob93 binding (mean ± SEM; n=3)
Control (MOPC)	3.4 ± 0.5
IV3 (anti-CD32) + MOPC	3.0 ± 0.4
Bob93	9.4 ± 1.8
IV3 + Bob93	6.8 ± 0.7

TABLE II
Effect of anti-FcγRII (CD32) on Bob93 binding to monocytes.
Cells were incubated on ice for 15 minutes with IV3 (10 µl; 50 µg/ml) before addition of Bob93 supernatant (40 µl) for a further 30 minutes. The cells were washed, incubated with 1:100 FITC labelled goat anti-mouse IgG1 for 30 minutes, washed again and analysed with an EPICS Profile II flow cytometer. The mean log fluorescence was recorded for each sample.
The nature of the inhibitory moiety in rabbit serum was investigated further by separating the proteins remaining in the IgG-depleted NRIG on an SDS-polyacrylamide gel and visualising the bands by silver

staining. This procedure revealed a dominant band with a MW of approximately 60 kD. Serum proteins that were candidates for the inhibitory factor were chosen on the basis of their molecular weight, and then tested for inhibition of Bob93 binding in a flow cytometric assay. It was found that the sialated bovine protein fetuin, a major component of foetal calf serum, was able to inhibit Bob93 binding to monocytes when used at a high concentration (10 mg/ml) (figure 7). At lower concentrations, fetuin augmented Bob93 binding, suggesting that the fetuin constitutively present in the Bob93 supernatant may have been acting as a molecular bridge between the cell and the Bob93 antibody. The effect of fetuin was specific since no effect on Bob93 binding was found following incubation with bovine albumin or asialofetuin.

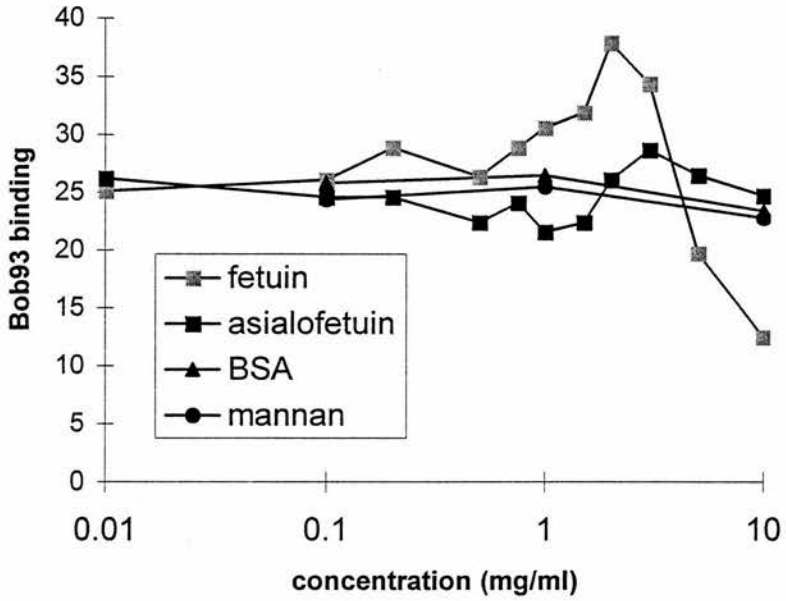


FIGURE 7
Fetuin acts as a molecular bridge between the Bob93 and the cell surface.
Binding of Bob93 supernatant to human monocytes was augmented by low concentrations and inhibited by high concentrations of fetuin. In contrast, BSA, asialofetuin, and mannan have no effect.

Fetuin is the Bob93 antigen

To determine whether fetuin was indeed acting as a molecular bridge, the Bob93 hybridoma cells were grown in a serum-free medium for 24 hours prior to collection of the supernatant. The serum-free Bob93 did not bind to monocytes or apoptotic neutrophils, but binding to both cell types could be reconstituted by addition of fetuin (figure 8). Bovine albumin and asialofetuin were unable to reconstitute binding, supporting the hypothesis that fetuin was a specific ligand for Bob93.

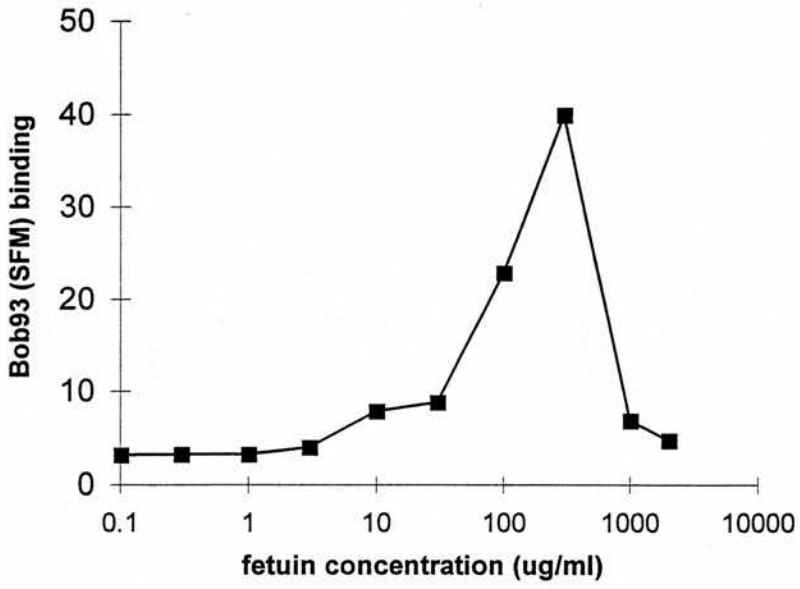


FIGURE 8
Binding of serum-free Bob93 is reconstituted by fetuin
Bob93 serum-free supernatant (SFM) was incubated with human monocytes in the presence of increasing concentrations of bovine fetuin. Bob93 binding was detected with indirect immunofluorescence and flow cytometry. Results from one representative experiment are shown.

Specific binding of Bob93 to fetuin and to fetal calf serum was confirmed by dot-blotting (figure 9). Interestingly, Bob93 did not bind to human serum or α_2-HS glycoprotein, the human homolog of bovine fetuin, or to rabbit serum.

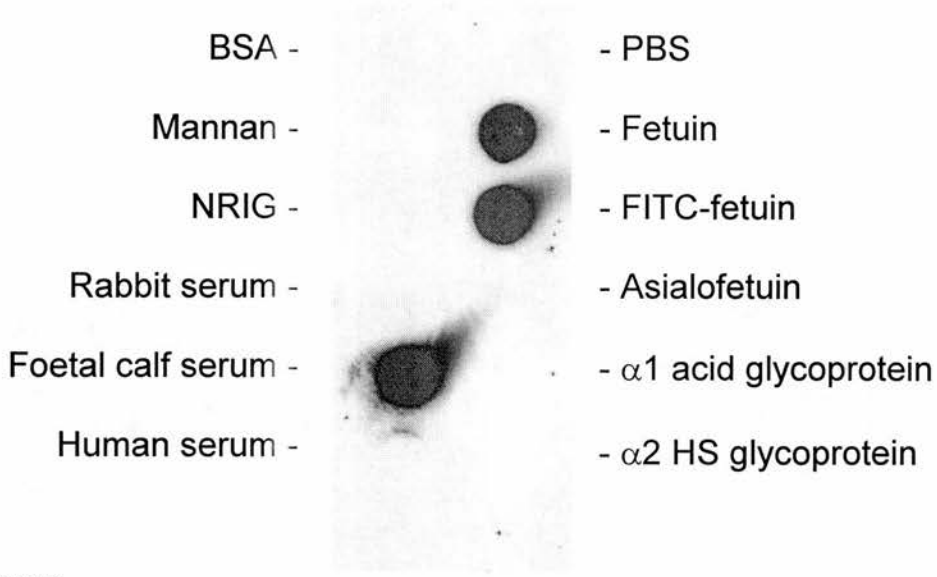


FIGURE 9
Bob93 binds specifically to fetuin.
Proteins (1 μg) were dot-blotted onto a nitrocellulose membrane and allowed to dry. The membrane was blocked with Tris-buffered saline containing 0.1% Tween 20 and then probed with 1:100 Bob93 hybridoma serum free supernatant followed by 1:4000 goat anti-mouse Ig-HRP. The membrane was developed using enhanced chemiluminescence (Amersham) or 0.5 mg/ml diaminobenzidine containing 0.03% H_2O_2 (not shown).

In summary, these data imply that Bob93 binds in a sialic acid-dependent and species-specific manner to bovine fetuin, and that fetuin may bind to the surface of both human monocytes/macrophages and apoptotic neutrophils.

Labelled fetuin binds to the cell surface

Fetuin is a sialated glycoprotein that was first isolated as the principal protein component of foetal calf serum. It contains complex oligosaccharides buts its core peptide displays significant homology between the many different species in which it has been identified. The principal physiological role of fetuin remains unknown, but it has been reported to be involved in cell growth (Nie, 1992; Ohnishi et al., 1997), fatty acid transport (Cayatte et al., 1990), bone mineralisation (Schinke et al., 1996), haematopoiesis (White et al., 1993), immune responses (Splitter and Everlith, 1982), monocyte phagocytosis (Lewis and Andre, 1981) and chemotaxis (Malone and Richards, 1987), TGF-β receptor binding (Demetriou et al., 1996), and protease inhibition (Yamamoto and Sinohara, 1993).

To determine whether fetuin can bind to the cell surface, purified protein was labelled with biotin or fluoroscein isothiocyanate (FITC) and cell binding was examined using flow cytometry. Fetuin bound in a concentration-dependent manner to mononuclear phagocytes but not to lymphocytes (figure 10).

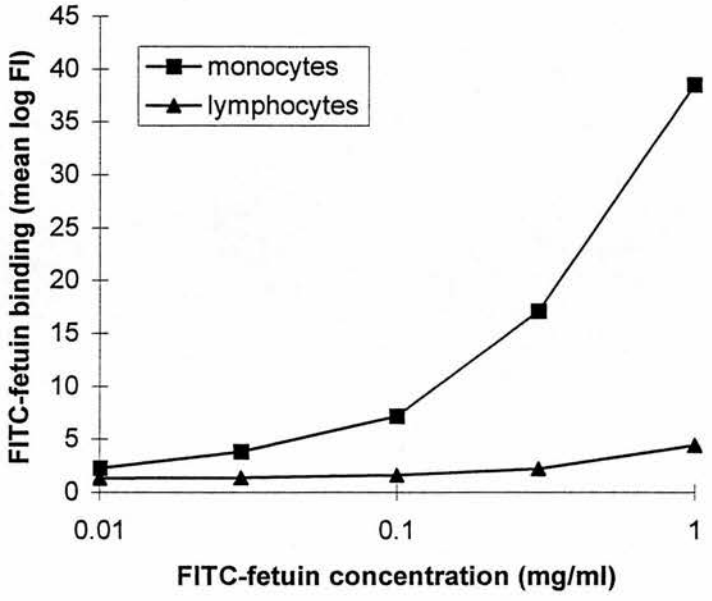


FIGURE 10
Labelled fetuin binds to monocytes but not lymphocytes.
FITC-labelled fetuin was incubated with cells for 30 minutes on ice before washing and flow cytometric analysis. Results from one representative experiment are shown.

Interestingly, addition of Bob93 serum-free supernatant augmented binding of fetuin to monocytes and macrophages, suggesting that the Bob93-fetuin complex had a higher affinity than fetuin alone for its putative receptor on the cell surface. One possibility was that augmented cell binding was a result of Bob93-induced crosslinking of fetuin that led to formation of a multimeric complex. However, the lack of effect of a polyclonal fetuin antibody implied that fetuin crosslinking was not responsible for the augmented cell binding seen with Bob93 (table III).

Antibody	FITC-fetuin binding to macrophages
None	6.3
Bob93	18.1
Anti-fetuin 1:10000	6.4
Anti-fetuin 1:1000	6.3
Anti-fetuin 1:100	6.2

TABLE III
Effect of co-incubation with fetuin antibodies on binding of FITC-labelled fetuin to macrophages.
Human monocyte-derived macrophages were incubated for 30 minutes with 0.4 mg/ml FITC-fetuin and 1:2 Bob93 serum free supernatant or polyclonal anti–fetuin (rabbit serum) at three different dilutions. Data are mean log fluorescence values from one experiment. In this experiment the mean LFL of unlabelled macrophages was 2.6. Both antibodies recognised fetuin in dot-blotting experiments.

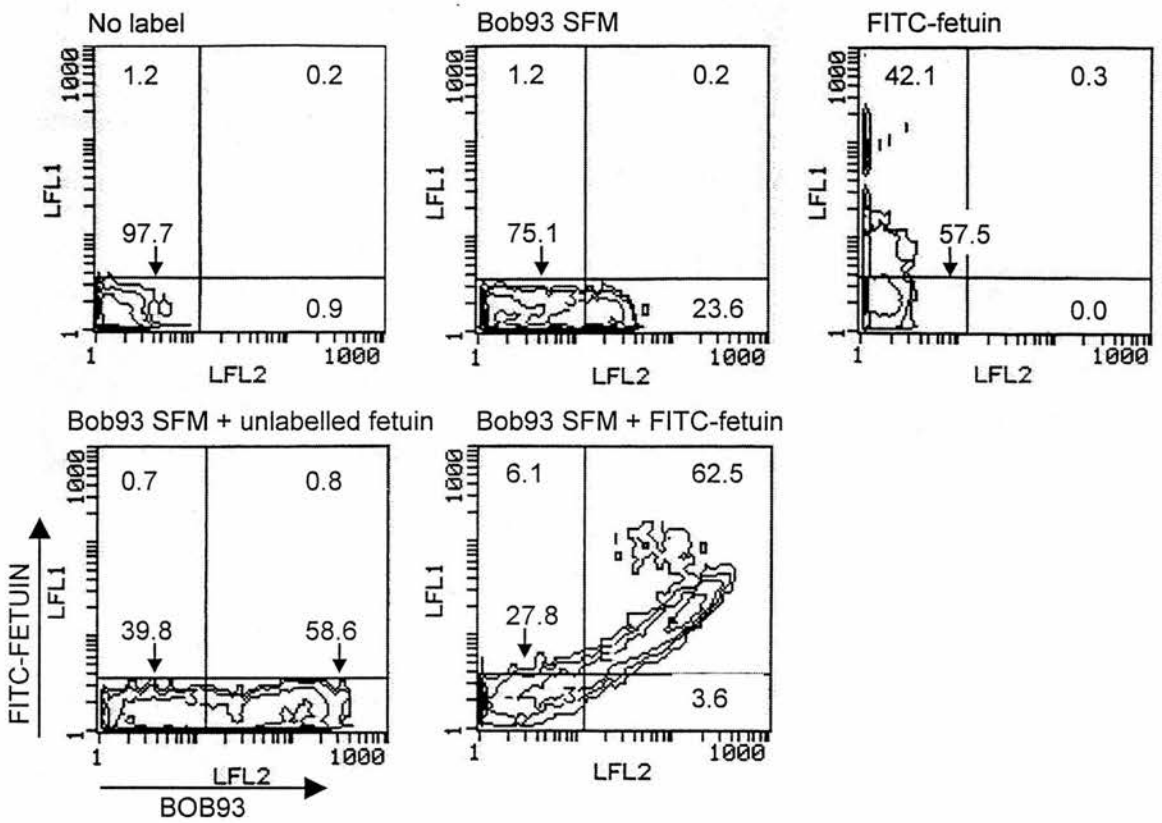


FIGURE 11

Dual colour flow cytometry profiles of Bob93 and fetuin binding to aged neutrophils

Aged human neutrophils were washed and incubated with combinations of buffer alone, 1:2 Bob93 in serum-free medium (SFM), 0.1 mg/ml unlabelled fetuin, or 0.1 mg/ml FITC-labelled fetuin. The cells were washed prior to further incubation with 1:25 goat anti-mouse-PE. The cells were washed again and analysed by flow cytometry. FL1 (y axis) represents binding of FITC-fetuin and FL2 (x axis) represents binding of Bob93. The percentage of cells in each quadrant is presented. FITC-fetuin binding is dependent on the presence of Bob93, and the same population of (apoptotic) cells is labelled by both markers.

Similarly, constitutive fetuin binding to the mouse macrophage cell line J774 was substantially increased by the addition of Bob93 (table IV).

Protein	Cell binding
Control	2.9 ± 0.7
Fetuin	7.6 ± 2.7
Fetuin + Bob93	33 ± 12.7

TABLE IV

Binding of biotinylated fetuin (0.4 mg/ml) to mouse J774 cells in the presence and absence of Bob93 (1:2 serum free supernatant). Values presented are means ± SEM of mean log fluorescence from three independent experiments.

To confirm that the augmentation of fetuin binding was due to the Bob93 antibody and not to another constituent of the serum free medium, the supernatant was depleted of Bob93 with fetuin-agarose or anti-mouse Ig-agarose. Both of these treatments significantly reduced the ability of Bob93 supernatant to augment fetuin binding (table V)

Treatment	Cell binding
Control	2.6
Fetuin	6.3
Fetuin + Bob93 (sham depleted)	16
Fetuin + Bob93 (depleted with anti-mouse Ig-agarose)	7.4
Fetuin + Bob93 (depleted with fetuin-agarose)	10.5

TABLE V
Depletion of Bob93 supernanant inhibits augmentation of fetuin binding.
Biotinylated fetuin binding to human monocyte-derived macrophages was assessed in the presence of Bob93 serum-free supernatant that had been pre-incubated with glycine-agarose (sham), anti-mouse Ig-agarose, or fetuin-agarose. Results are mean log fluorescence values from one experiment.

Fetuin does not bind to apoptotic neutrophils in the absence of Bob93

The nature of Bob93 binding to the different cell types examined so far had seemed indistinguishable, and although Bob93 binding to mononuclear phagocytes was an interesting finding in itself, one of the reasons for using monocytes and macrophages was as convenient surrogates for apoptotic neutrophils. The demonstration that fetuin was the ligand for Bob93 raised the exciting possibility that a serum glycoprotein could bind to apoptotic but not non-apoptotic neutrophils. However, in contrast to mononuclear phagocytes which exhibited constitutive fetuin binding that could be augmented by Bob93, binding of fetuin to apoptotic neutrophils was demonstrable only in the presence of Bob93.

No constitutive binding could be demonstrated following manipulations of pH or cation concentrations, using different media for the incubation, or reducing the stringency of the washing step. That fetuin binding to apoptotic neutrophils was dependent on the presence of Bob93 again raised intriguing questions about the mechanism of Bob93-augmented binding of fetuin to mononuclear phagocytes. Evidence has been presented that simple crosslinking of fetuin was not responsible, and this was supported by the observation that apoptotic neutrophils failed to bind fetuin-coated latex beads or to plastic surfaces coated with fetuin.

Together, these observations raised the possibility that the fetuin-Bob93 interaction may mask sialic acid residues that result in reduced repulsion between fetuin and the cell surface. If this were the case then asialofetuin, the desialated derivative of fetuin, should bind to cells more strongly than fetuin. However, biotinylated asialofetuin did not bind to apoptotic neutrophils, although weak binding to monocytes could be demonstrated (table VI).

Labelled protein	Binding to cell surface
None	1.8
Asialofetuin	5.7
Fetuin	29.7

TABLE VI
Binding of biotinylated asialofetuin and fetuin to the human monocytes.
Labelled glycoproteins (0.4 mg/ml) were incubated with monocytes and cell binding was assessed by flow cytometry. Mean log fluorescence values are presented from one experiment.

One explanation for the different characteristics of fetuin binding to mononuclear phagocytes and apoptotic neutrophils is that fetuin is not the true physiological ligand, but is simply an experimental substitute for another sialated glycoprotein that may bind to both cell lineages. Indeed, fetuin is a bovine protein, and preliminary experiments to date suggested that its human homolog, α2 HS glycoprotein, did not function in a similar way in in vitro studies. In particular, α2 HS glycoprotein did not compete with labelled fetuin for cell binding sites as unlabelled fetuin did, and it seemed to exert no effect on macrophage phagocytosis of apoptotic neutrophils. However, these experiments were hampered by the high cost of commercially available α2 HS glycoprotein compared with fetuin (£80 compared with £1 per milligram; 1998 prices).

Specific binding of labelled fetuin

To demonstrate specific binding of fetuin to the cell surface, binding of labelled fetuin was competed by co-incubation with higher concentrations of unlabelled fetuin or other glycoproteins. Approximately 50% of labelled fetuin binding was specific (figure 12).

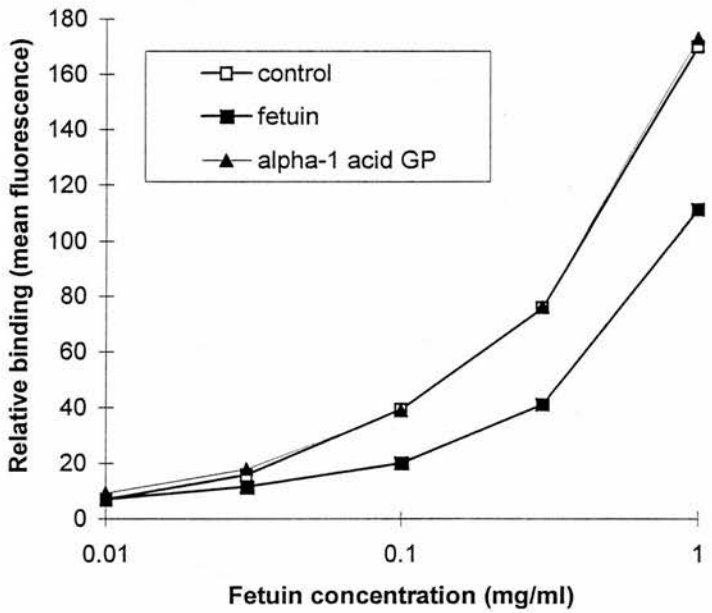


FIGURE 12
Specific binding of labelled fetuin
Binding of fetuin to monocytes was competed with 5 mg/ml unlabelled fetuin or alpha-1 acid glycoprotein (control).

The lack of fetuin binding to apoptotic neutrophils fuelled the search for the true physiological glycoprotein for which fetuin was simply an experimental surrogate. A variety of glycoproteins were tested in competition assays with labelled fetuin. Interestingly, pre-incubation of cells with the human sialoglycoprotein lactoferrin actually *increased* fetuin binding to human monocytes, macrophages, or mouse J774 cells (table VII).

	Macrophage (n=1)	THP-1 (n=1)	J774 (n=4)
Control (2nd layer only)	2.8	2.3	2.8 ± 0.5
Labelled fetuin	9.3	4.2	7.6 ± 1.9
Lactoferrin + labelled fetuin	17.7	3.8	84.9 ± 40.5

TABLE VII
Effect on fetuin binding of pre-incubation of cells with human lactoferrin.
Human macrophages or murine J774 cells were incubated with 1 mg/ml human lactoferrin or 1 mg/ml BSA (control) on ice for 30 minutes. Cells were washed twice before analysis of biotinylated fetuin binding as previously described. Results are mean ± SEM of mean log fluorescence.

However, lactoferrin had no effect on fetuin binding to apoptotic neutrophils. The reason for this differential effect became clear when binding of biotinylated lactoferrin bound to cells was examined. Labelled lactoferrin bound strongly to mononuclear phagocytes, but not to apoptotic neutrophils (figure 13).

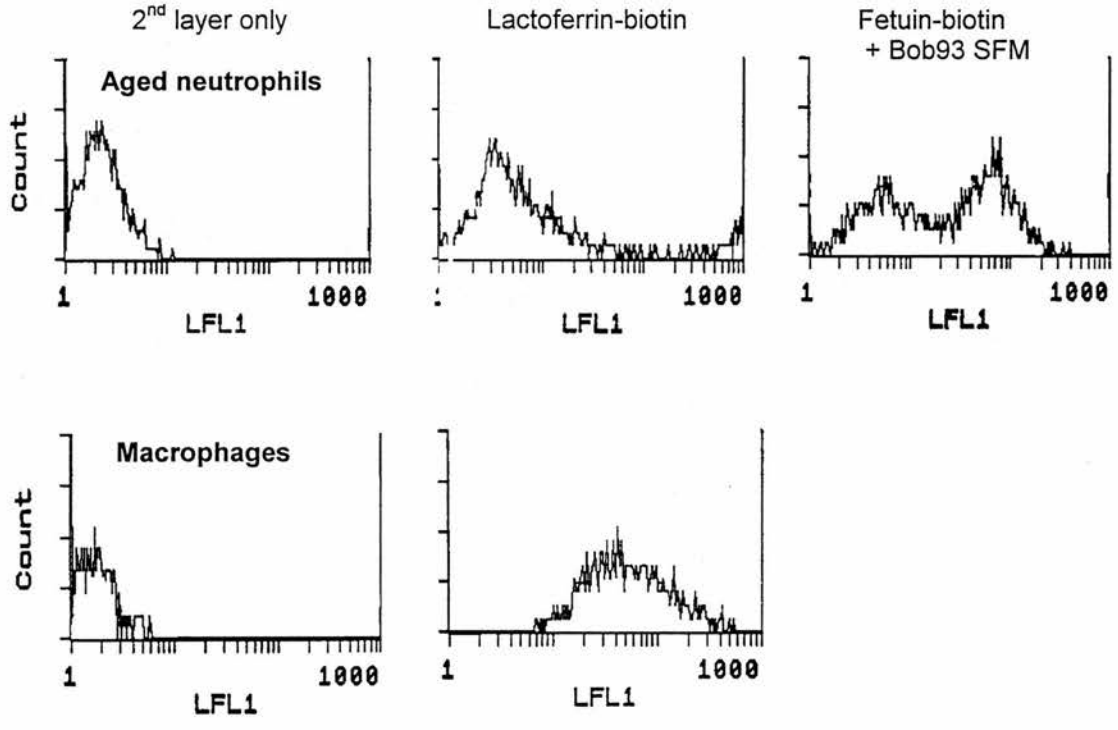
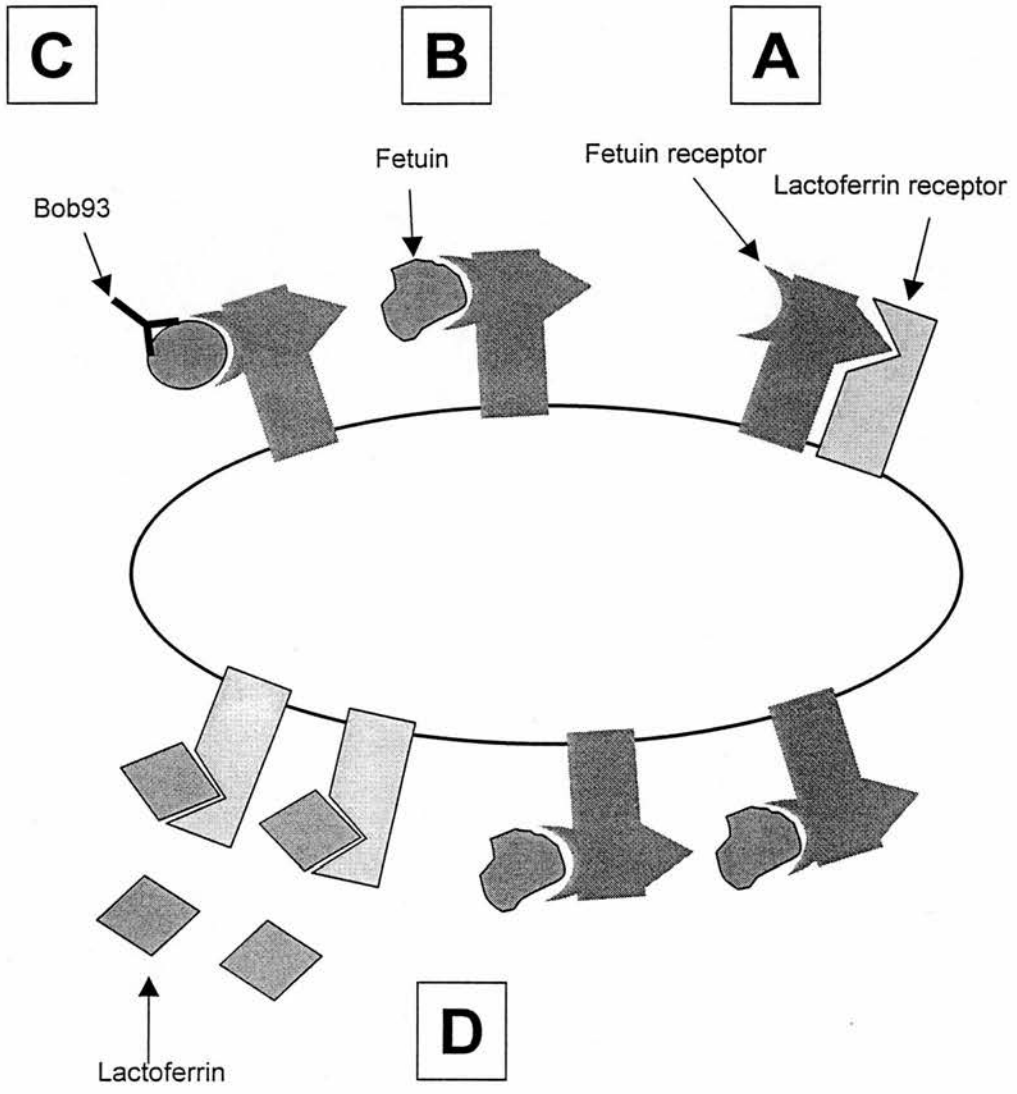


FIGURE 13
Lactoferrin binds to macrophages but not to aged neutrophils
Human macrophages or aged neutrophils were incubated with 1 mg/ml biotinylated human lactoferrin followed by streptavidin-FITC second layer. Lactoferrin bound only to macrophages and a small number of membrane permeable, necrotic neutrophils present within the population of aged cells. In contrast, fetuin-Bob93 bound to apoptotic neutrophils.

Dot blot and affinity isolation studies suggested that there was no direct interaction between fetuin and lactoferrin. These observations suggested that binding of lactoferrin to the mononuclear phagocyte surface induced some alteration in the putative fetuin receptor that increased its affinity for fetuin.

Analysis of lactoferrin binding and its effect on subsequent cell binding of fetuin permitted the generation of a model which is shown in figure 14.



KEY

A. In the resting state, the fetuin receptor may be associated with a lactoferrin receptor. Compared with macrophages, a much higher proportion of J774 fetuin receptors are occupied in this way, so that J774 cells exhibit lower basal fetuin binding. Neutrophils do not possess a lactoferrin receptor.

B. Fetuin binds to its receptor on macrophages or J774 cells. Neutrophils posses a fetuin receptor but do not exhibit constitutive fetuin binding..

C. Monoclonal antibody Bob93 induces a conformational change in fetuin that increases its affinity for the receptor. In neutrophils, the presence of Bob93 is required for fetuin binding. The lack of effect of a polyclonal fetuin antibody suggests that cross-linking is not sufficient. The failure of asialofetuin to bind significantly to cells means that masking of sialic acid residues cannot explain this phenomenon.

D. Lactoferrin displaces the fetuin receptor from its binding site on the lactoferrin receptor, so increasing the number of fetuin binding sites on the cell.

FIGURE 14
Model of the interaction between cells, Bob93, fetuin, and lactoferrin.

Affinity isolation of fetuin-binding proteins

The flow cytometric analysis of fetuin binding to cells suggested that there may be specific receptors on the cell surface for fetuin or fetuin-like proteins. To investigate this possibility further biotin-labelled cell surface proteins were identified on Western blots following solubilisation with 1% NP-40 and affinity isolation with fetuin-agarose (figure 15). Human macrophages demonstrated strong bands at 35 kD, whereas 35 and 40 kD bands from J774 cells were reproducibly isolated. Apoptotic human neutrophils showed a weak band at ~35 kD that was not present in isolates from aged but non-apoptotic neutrophils (stored at 4°C for 20 hours rather than 37°C)(figure 16). There were no surface labelled bands from isolates from human THP-1 cells or peripheral blood lymphocytes, which also fail to exhibit fetuin binding in flow cytometry (figure 16).

The pattern of surface labelled proteins could be compared with the total proteins isolated by fetuin affinity isolation by staining the nitrocellulose blot with colloidal gold. In addition to the surface labelled proteins, colloidal gold staining revealed identical strong bands of 115 kD and 55 kD from all cell types (figure 17), presumably representing intracellular proteins such as sialotransferases.

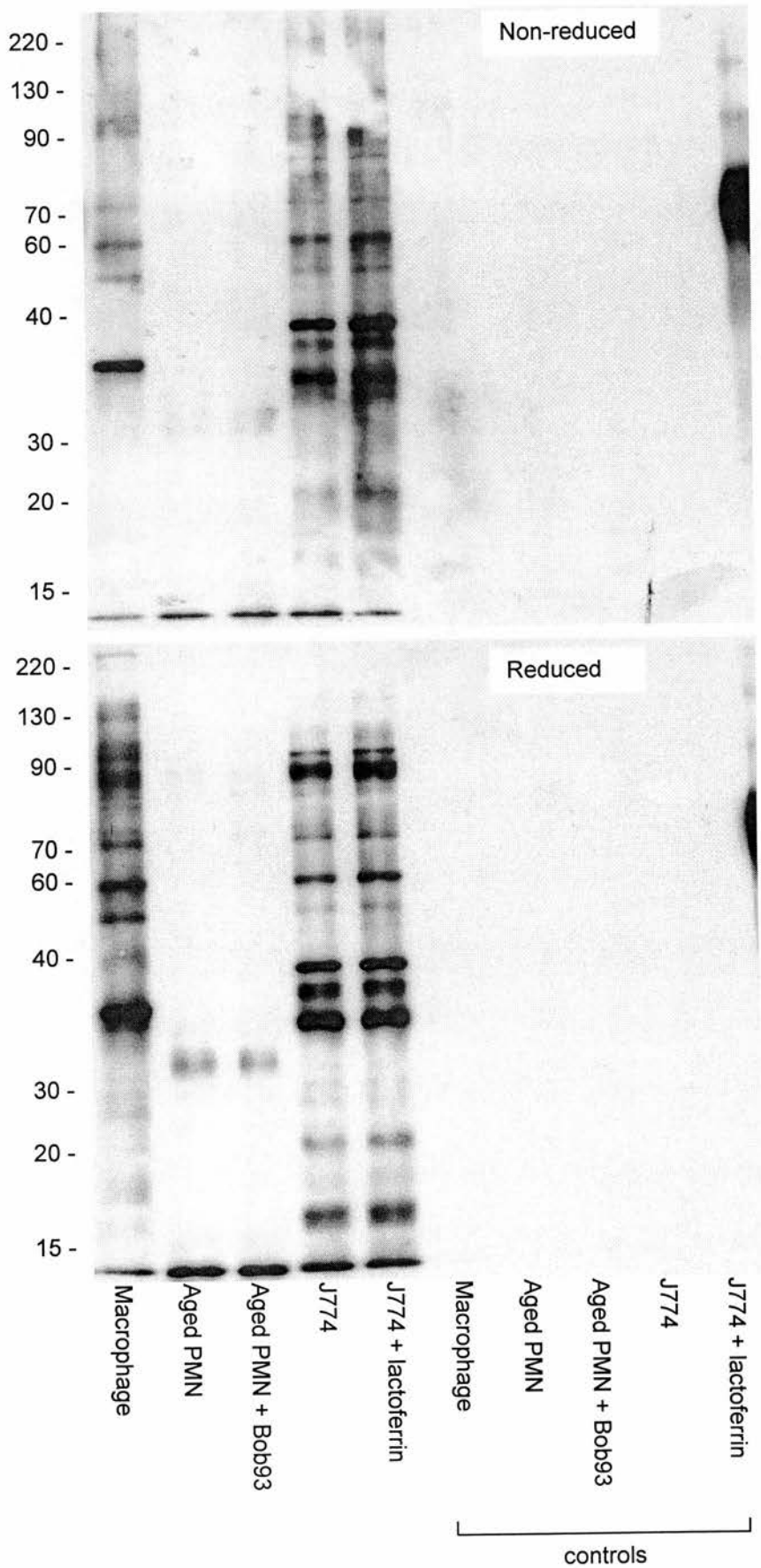

Non-reduced
Reduced
220 -
130 -
90 -
70 -
60 -
40 -
30 -
20 -
15 -
Macrophage
Aged PMN
Aged PMN + Bob93
J774
J774 + lactoferrin
Macrophage
Aged PMN
Aged PMN + Bob93
J774
J774 + lactoferrin
controls

FIGURE 15 (PREVIOUS PAGE)
Affinity isolation of surface-labelled fetuin-binding proteins
Cells were surface labelled with biotin and lysed in 1% NP-40 lysis buffer containing protease inhibitors. The NP-40-soluble lysates were incubated in the presence or absence of 1:2 Bob93 SFM or 0.1 mg/ml lactoferrin with fetuin-agarose or glycine-agarose (controls) and the pellets washed five times with lysis buffer. Fetuin-binding proteins were eluted from the agarose pellets by boiling in non-reducing (upper panel) or reducing (lower panel) 4% SDS sample buffer, and samples were run on a 10% polyacrylamide gel and blotted onto a nitrocellulose membrane. The membrane was blocked and surface-labelled fetuin binding proteins were identified by incubation with 1:5000 streptavidin-HRP (Dako) and enhanced chemiluminescence (Amersham).

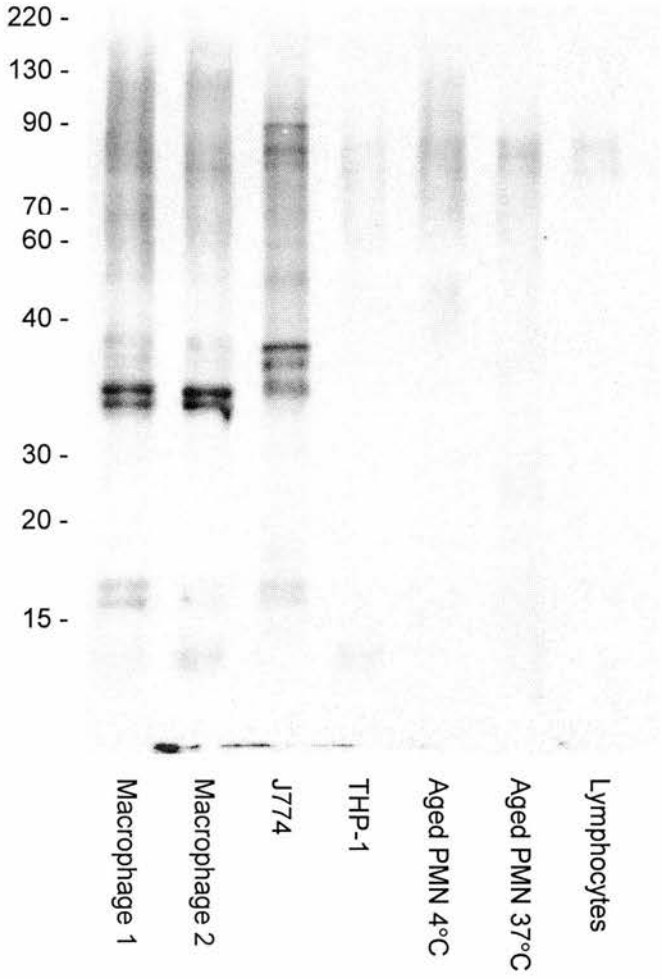


FIGURE 16
Cell specificity of fetuin affinity isolation
Surface labelled fetuin binding proteins could be isolated from macrophages and J774 cells, but not from THP-1 cells, human peripheral blood lymphocytes, or neutrophils aged for 20 hours at 4°C or 37°C.

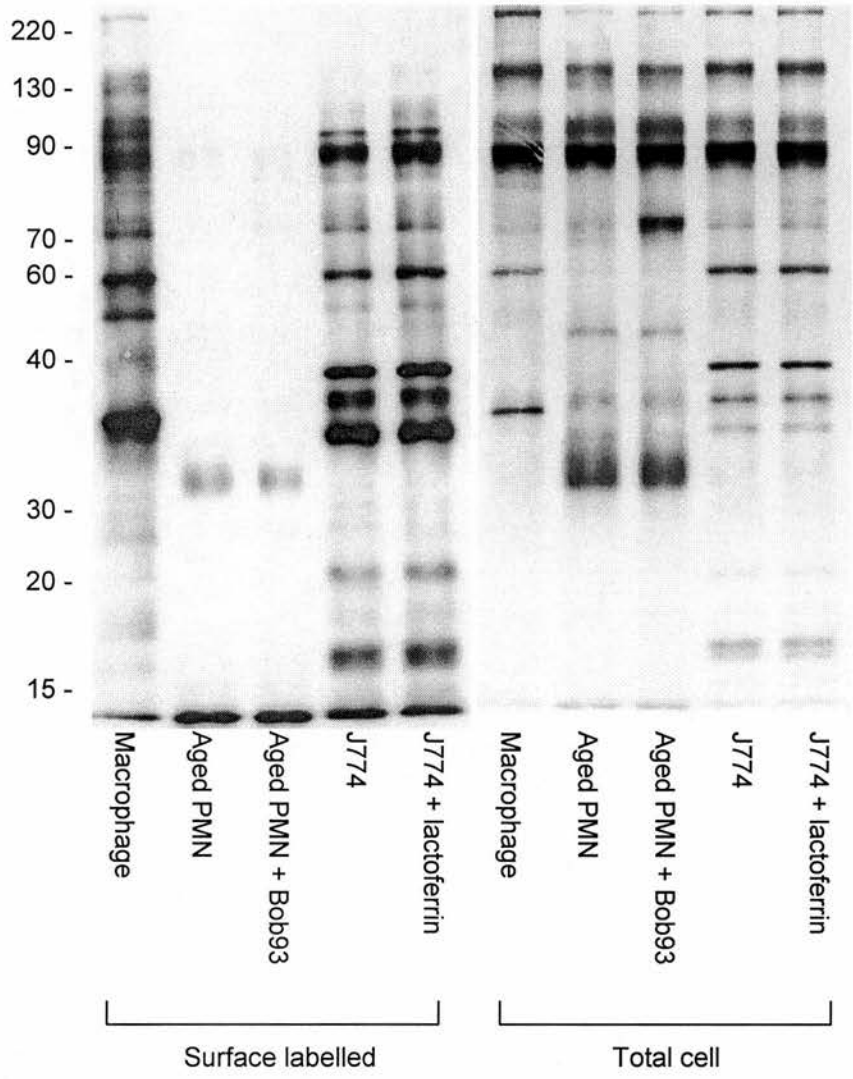


FIGURE 17
Comparison of surface-labelled and total cell detergent-soluble fetuin binding proteins
The nitrocellulose blot of reduced fetuin binding proteins shown in figure 9 (lower panel) was washed and incubated with colloidal cold (Pierce) to stain all proteins on the blot. Compared with surface labelled proteins (left panel), this analysis revealed additional bands of 85-90 kD in from the aged neutrophil lysates that appeared identical to bands from macrophages and J774 cells.

Fetuin enhances macrophage phagocytosis of apoptotic neutrophils

Fetuin is present at high concentrations in the serum of the foetus (Dziegielewska et al., 1993), which is able to repair damaged tissue without scarring. In the adult, serum concentrations of human fetuin may be altered in a variety of inflammatory diseases (Lebreton et al., 1979). These observations suggested that fetuin could play a role in the inflammatory response. Phagocytic clearance of apoptotic neutrophils is likely to be a key process in the resolution of inflammation, and a role for fetuin could be postulated since fetuin bound to the plasma membrane of both macrophages and apoptotic neutrophils. Therefore the effect of fetuin on human macrophage phagocytosis of apoptotic neutrophils was examined using the well-characterised in vitro phagocytosis assay.

Fetuin specifically enhanced macrophage phagocytosis of apoptotic neutrophils by two to three fold during a 30 minute interaction (Figure 18). Both the percentage of macrophages that ingested one or more apoptotic neutrophils, and the phagocytic index (number of ingested neutrophils per 100 macrophages) were increased by fetuin. There was a small effect with asialofetuin, and albumin and α1-acid glycoprotein demonstrated an inhibitory effect compared with medium alone. Soluble fetuin may bind to the macrophage surface where it is immobilised and presented in a form that is bound by the apoptotic neutrophils, whereupon a phagocytic signal is delivered to the macrophage. This model is consistent with previous reports that human monocytes are able to ingest latex beads in the presence of α2-HS glycoprotein (Lewis and Andre, 1981), the human homolog of bovine fetuin.

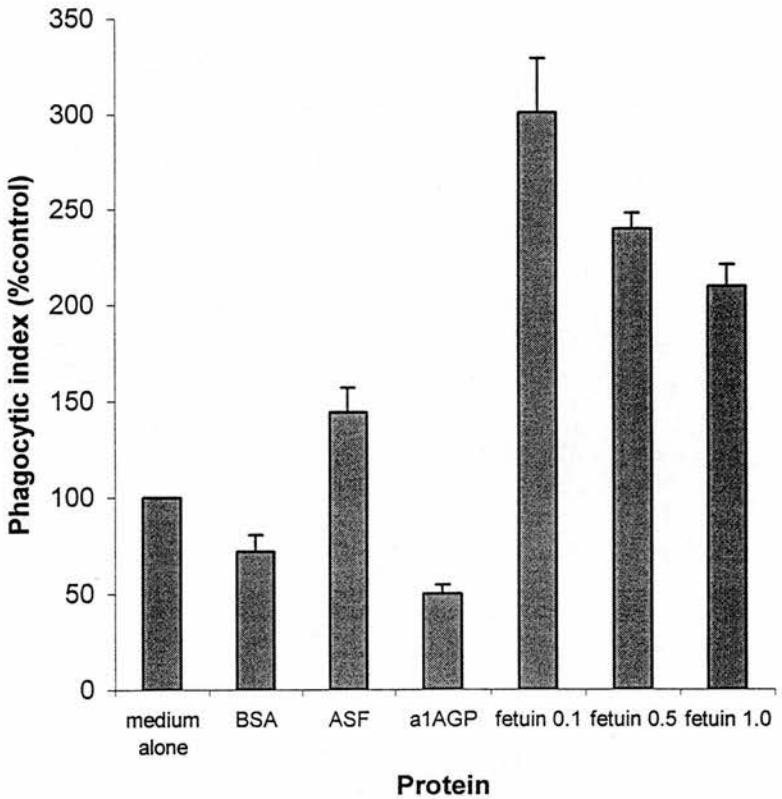


FIGURE 18
Fetuin specifically enhances human macrophage phagocytosis of apoptotic neutrophils.
Fetuin (0.1-1.0 mg/ml) or control proteins (0.5 mg/ml) were pre-incubated with adherent human macrophages for 30 minutes at room temperature prior to addition of apoptotic human neutrophils. Following incubation for 30 minutes at 37°C the wells were washed to remove non-ingested cells, fixed in glutaraldehyde, and neutrophil peroxidase activity was stained with H_2O_2/DMB (a1AGP, α1 acid glycoprotein; ASF, asialofetuin; BSA, bovine serum albumin).

SUMMARY

Down-regulation of a variety of surface molecules is associated with neutrophil apoptosis, which is likely to contribute to the characteristic functional isolation of these dying cells. Subtle changes in the

carbohydrate composition of surface molecules may alter the net negative charge of the cell that influences adhesive interactions with phagocytes. In contrast, very few "positive" surface changes on the apoptotic cell surface have been identified to date. In the present study a monoclonal antibody, Bob93, which binds specifically to mononuclear phagocytes and apoptotic neutrophils but not to other apoptotic leukocytes, has been characterised. I have presented preliminary evidence that the antigen recognised by Bob93 is present on the bovine sialoglycoprotein fetuin, which may act as a molecular bridge to enhance phagocytosis of apoptotic neutrophils by macrophages. Serum concentrations of human fetuin (α2-HS glycoprotein) may fall during inflammatory diseases, an observation that lead to the proposal that fetuin represents a negative acute phase reactant (Lebreton et al., 1979). Further support for the suggestion that fetuin plays a role in inflammation and repair comes from the demonstration that fetuin is present in very high concentrations in the serum of foetus (Dziegielewska et al., 1993), which displays the remarkable ability to heal without scarring. Clearance of apoptotic neutrophils is an important process in the resolution of inflammation, and it has been demonstrated in this chapter that fetuin may modulate macrophage phagocytosis of apoptotic neutrophils in vitro. Altered production of fetuin and its homologs during inflammatory processes is likely to represent a further mechanism for the regulation of phagocytosis of apoptotic neutrophils and hence determine the outcome of inflammation.

CHAPTER 6
CONCLUDING REMARKS

It has been hypothesised that inadequate phagocytosis of apoptotic cells by macrophages may contribute to the tissue damage and perpetuation of inflammation that characterise a variety of chronic diseases. Recent experimental evidence supports such a role for overwhelming apoptosis and failed clearance in such adverse tissue outcomes. Many aspects of macrophage behaviour, including phagocytosis, are modulated by interactions of cells with the extracellular matrix via ligation of specialised adhesion receptors and subsequent transduction of intracellular signals. CD44 is a receptor that has been implicated in adhesion to a variety of matrix components, and may be associated with cytoskeletal proteins and intracellular signalling pathways. Furthermore, CD44 has been proposed to play a role in the regulation of macrophage function in inflammation. In this thesis it has been demonstrated that phagocytosis of apoptotic neutrophils, but not apoptotic lymphocytes, by human monocyte-derived macrophages is rapidly augmented following ligation of CD44 by bivalent antibodies in vitro. These observations, together with the lack of effect of CD44 antibodies upon macrophage phagocytosis of zymosan or immunoglobulin-opsonized erythrocytes imply that CD44 may regulate the differential clearance of apoptotic leukocytes during evolution of inflammatory responses.

The data presented here suggest that macrophage CD44 ligation results in recruitment of a new molecular mechanism for removal of apoptotic neutrophils that is much less dependent on the presence of divalent cations than the pathway that operates under basal conditions. The apoptotic cell receptor mechanism recruited following CD44 ligation was, like the basal recognition mechanism, sensitive to broad spectrum proteases and cytoskeletal inhibitors, but did not appear to be GPI-linked to the macrophage membrane. Experiments using previously defined inhibitors demonstrated that CD44-augmented phagocytosis was unlikely to be mediated by classical apoptotic cell recognition receptors such as $\alpha v\beta 3$, lectins, the phosphatidylserine receptor, scavenger receptor, or the 61D3 epitope of CD14. In addition, I have used monoclonal antibodies and competitive ligands to rule out a role for other cell adhesion molecules such as $\beta 2$ integrins or sialoadhesin. The precise molecular pathway(s) recruited following CD44 ligation remains to be defined. Rather than continuing to screen individual candidate receptors using inhibitory ligands or antibodies, it seemed more promising to pursue the underlying regulatory mechanism by analysis of the intracellular molecular events and the functional responses that occurred following macrophage CD44 ligation. The validity of this approach was supported by the finding that $F(ab')_2$ fragments of CD44 antibody were capable of augmenting phagocytosis but that monovalent Fab' fragments had no effect. That receptor cross-linking was a prerequisite for the response is further supported by recent data showing that cross-linking of CD44 Fab' fragments with secondary antibodies results in augmented phagocytosis of apoptotic neutrophils similar to that seen with intact CD44 antibody {K. Ross and I. Dransfield, unpublished observations).

Analysis of macrophage release of pro-inflammatory cytokines following CD44-augmented phagocytosis of apoptotic neutrophils revealed very low concentrations of TNF-α in the culture supernatant. This

observation was attributed to low levels of macrophage TNF-α secretion, although sequestration by specific TNF-α receptors or surface carbohydrate chains could not be excluded. Further experiments looking at synthesis or intracellular stores of TNF-α were not performed, since in this context only release of active cytokine was considered important. Similar experiments with IL-8 were more revealing, with macrophages demonstrating a clear stimulation of IL-8 release in response to zymosan, Fc-receptor mediated phagocytosis, or LPS, but no demonstrable response to ingestion of apoptotic neutrophils either with or without preceding CD44 antibody treatment. This finding is consistent with a model of apoptotic cell recognition as a "silent" or even anti-inflammatory process. Analysis of IL-10 release again revealed principally undetectable concentrations in macrophage supernatants, which may have been a result of low levels of release or of sequestration at the cell surface. Further studies might explore the possibility that the repertoire of macrophage cytokine production is altered following engulfment of apoptotic neutrophils with or without preceding CD44 antibody treatment.

The rapidity of effect of CD44 antibodies suggested that ligation of macrophage CD44 by antibodies may trigger intracellular signalling pathways that lead to increased phagocytosis of apoptotic neutrophils. Although CD44 has been reported to be associated with tyrosine kinases in lymphocytes, treatment with CD44 antibody had no effect on the pattern of major tyrosine-phosphorylated proteins in human monocyte-derived macrophages, although this is an insensitive method for examining phosphorylation of minor proteins. Immunoprecipitation of paxillin from macrophage lysates prior to Western blotting with phosphotyrosine antibody demonstrated that CD44 ligation resulted in increased tyrosine phosphorylation of paxillin. However, the importance of tyrosine phosphorylation of paxillin following CD44 ligation in relationship to augmented phagocytosis of apoptotic neutrophils is unclear, since the broad spectrum tyrosine kinase inhibitor genestein inhibits a variety of cellular processes, including phagocytosis of apoptotic neutrophils and Immunoglobulin-opsonized particles. Furthermore, paxillin is involved in a number of cytoskeletal events including integrin-mediated adhesion. It is unclear whether CD44-mediated tyrosine phosphorylation of paxillin is causally related to the apparently specific augmentation of macrophage phagocytosis of apoptotic neutrophils, or whether it is simply an epiphenomenon. Determination of paxillin localisation within the macrophage and its association with other cytoskeletal and membrane proteins, and functional studies with specific cell-permeable peptide inhibitors, would allow further dissection of the relationship between tyrosine phosphorylation of paxillin and enhanced phagocytosis of apoptotic neutrophils following CD44 ligation.

The role of CD44 variant isoforms has been examined briefly, but a more detailed analysis using antibodies against a diverse range of variants would be required to confirm or refute a role in phagocytosis of apoptotic cells. It would be especially interesting to examine the expression and function of those isoforms (CD44v4, v6, v9) that may be differentially expressed by macrophages at sites of inflammation in vivo.

CD44 has been reported to bind to a diversity of ligands which may exert differing effects on macrophage function through binding to the same receptor. This principal is illustrated by the importance of fragment size on the stimulation of cytokine production by hyaluronan (Noble et al., 1993). The response of

macrophages to purified ligands in vitro, either in solution or adherent to plastic, may allow identification of one or more substances that mimic the effect of CD44 antibody on phagocytosis of apoptotic neutrophils. This would lead naturally to investigation of the role of such ligand(s) in inflammatory responses in vivo. Furthermore, the mechanisms of induction of different CD44-mediated functions following binding to the extracellular domain of the receptor could be explored.

Subsequent studies of the mechanisms and roles of CD44-augmented phagocytosis would benefit from the use of chimeric CD4/CD44 molecules, which could be transfected into macrophages (C. Isacke, personal communication). This method would allow analysis of specific domains of the CD44 molecule. Similarly, transfection of CD44 mutants into CD44-negative cells in a "gain of function" approach could yield useful information. For example, the effect of wild type CD44 could be compared with CD44 mutants lacking the cytoplasmic ezrin-binding site or phosphorylation sites (C. Isacke, personal communication). However, such experiments could be hampered since the cells chosen (e.g., melanoma cells) may lack the essential machinery for phagocytosis. In addition to phagocytosis, analysis of other macrophage functions, such as antigen presentation, may also be influenced following CD44 ligation. CD44 has been reported to down-regulate inflammation in animal models of arthritis (Mikecz et al., 1995; Verdrengh et al., 1995), which has been attributed to altered leukocyte recruitment to the inflammatory site. However, it is feasible that CD44-mediated apoptotic cell clearance may have contributed to the anti-inflammatory effect observed in these experiments. The availability of a genetically manipulated CD44-deficient mouse (Schmits et al., 1997) offers additional potential for investigating the role of CD44 in inflammatory responses in vivo.

The molecular alterations on the surface of the apoptotic neutrophil that are responsible for phagocyte recognition have yet to be clearly characterised. This thesis describes the characterisation of Bob93, a new monoclonal antibody that binds specifically to apoptotic neutrophils. The antigen recognised by Bob93 is present on the bovine sialoglycoprotein fetuin. Fetuin alone fails to bind to apoptotic neutrophils, but can be induced to do so in the presence of the Bob93 antibody, an effect that appears to be independent of fetuin cross-linking. It is hypothesised that in these experiments fetuin is simply a surrogate for an as yet unidentified human glycoprotein that may constitutively bind apoptotic neutrophils. However, a series of experiments suggest that fetuin may act as a molecular bridge to enhance phagocytosis of apoptotic neutrophils by human macrophages in vitro. Further analysis of fetuin and its homologs may provide novel insights into both the molecular processes involved in neutrophil apoptosis and the regulation of apoptotic neutrophil phagocytosis in vivo.

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